

COMPOSITIONS AND METHODS FOR THE PRODUCTION OF BETAINE LIPIDS

This application is a Continuation-In-Part of co-pending U.S. Patent Appln. No. 10/118,495, filed April 8, 2002, which claims priority to U.S. Patent Appln. No. 60/283,812, 5 filed April 13, 2001.

This invention was made in part with government support under grant MCB-0109912, from the National Science Foundation. As such, the United States Government has certain rights in the invention.

10 FIELD OF THE INVENTION

The present invention relates to compositions and methods for the production of betaine lipids. The methods of the present invention comprise the expression of recombinant enzymes (e.g. from *Rhodobacter sphaeroides*) in host cells (e.g. bacteria, yeast and plants) to produce betaine lipid compounds including, but not limited to, Diacylglycerol-*O*-4'-(*N,N,N*-trimethyl) 15 homoserine (DGTS).

BACKGROUND

The ability to sustain conventional agriculture is based upon a high input of agrochemicals, such as phosphate-containing fertilizers. Conventional inorganic phosphorous 20 fertilizers may cause an inadvertent addition of heavy metals, which are contained as impurities.

For example, an analysis of phosphate fertilizers commonly used in Argentina was performed to determine the concentrations of heavy metals (such as chromium, cadmium, copper, zinc, nickel, and lead) found therein. L. Guiffre de Lopez Camelo *et al.*, "Heavy Metals Input with Phosphate Fertilizers used in Argentina," *Sci. Total Environ.*, 204(3): 245-250 25 (1997). The analysis revealed that: rock phosphate fertilizers contain the highest levels of cadmium and zinc; diammonium phosphate fertilizers contain enhanced levels of chromium; while superphosphate fertilizers contain the highest levels of copper and lead. *Id.* Thus, the continuous fertilization of soils could increase the heavy metal contents exceeding natural abundances in soils, and result in the transfer of these metals to the human food chain. *Id.*

30 Moreover, agricultural phosphate overfertilization creates environmental problems (e.g. contamination of water) and will lead to a depletion of naturally occurring phosphate fertilizer

resources in the near future. Therefore, it is highly desirable to develop new strategies to reduce the amount of phosphate fertilizer needed for the optimal growth of crop plants.

SUMMARY OF THE INVENTION

5 The present invention relates to compositions and methods for the production of betaine lipids. In one embodiment, the compositions of the present invention comprise the nucleic acids defined by SEQ ID NO: 1, SEQ ID NO: 2, or portions thereof. In one embodiment, the methods of the present invention comprise the expression of recombinant enzymes from *Rhodobacter sphaeroides* in host cells such as bacteria and plants to produce betaine lipid compounds
10 including, but not limited to, Diacylglyceryl-*O*-4'-(*N,N,N*-trimethyl) homoserine (DGTS).

In one embodiment, the present invention contemplates a composition comprising isolated and purified DNA having an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. In another embodiment, a composition comprising isolated and purified DNA having an oligonucleotide sequence selected
15 from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 23, and portions thereof, is contemplated.

It is not intended that the present invention be limited to deoxyribonucleic acids defined by SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. In another embodiment, the present invention contemplates a composition comprising ribonucleic acid (RNA) transcribed from the
20 DNA defined by SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

The present invention also contemplates a composition comprising protein translated from the ribonucleic acid (RNA) that was transcribed from the DNA defined by SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. In an alternative embodiment, the present invention contemplates a composition comprising antibodies produced from the protein translated from
25 the ribonucleic acid (RNA) that was transcribed from the DNA defined by SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

The present invention also contemplates vectors comprising SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. In one embodiment, said vector is selected from the group consisting of pQE-31, pACYC-31, pBlueScript II SK+, pPCR-Script Amp, and pYES2.

30 The present invention also contemplates host cells comprising vectors comprising the DNA defined by SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. In one embodiment,

the present invention contemplates a variety of host cells selected from the group consisting of *E. coli*, *R. sphaeroides*, *M. loti* and *A. thaliana*. In another embodiment, the present invention comprises transgenic plants comprising vectors comprising the DNA defined by SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

5 The present invention also contemplates a composition comprising isolated and purified DNA encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof.

It is not intended that the present invention be limited to isolated and purified deoxyribonucleic acids (DNA) encoding a protein having the amino acid sequence selected from 10 the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof. In another embodiment, the present invention contemplates a composition comprising ribonucleic acid (RNA) transcribed from DNA encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof.

In an alternative embodiment, the present invention contemplates a composition 15 comprising antibodies produced from the protein translated from the ribonucleic acid (RNA) that was transcribed from DNA encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof.

The present invention also contemplates vectors comprising the DNA encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ 20 ID NO: 4, or portions thereof. In one embodiment, the present invention contemplates a composition comprising a vector selected from the group consisting of pQE-31, pACYC-31, pBlueScript II SK+, pPCR-Script Amp, and pYES2.

The present invention also contemplates a variety of host cells comprising vectors 25 comprising the DNA encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof. In one embodiment, the present invention contemplates a host cell selected from the group consisting of *E. coli*, *R. sphaeroides*, *M. loti*, and *A. thaliana*. In another embodiment, the present invention comprises transgenic plants comprising vectors comprising DNA encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ 30 ID NO: 4, and portions thereof.

The present invention also contemplates variants of the amino acid sequences selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof. In one embodiment, the present invention contemplates variants of the *R. sphaeroides* BtaA peptide defined by the amino acid sequence of SEQ ID NO: 3, wherein said variant comprises an amino acid substitution selected from the group consisting of:

T2S; A5G; L6I; T7S; L9I; A11G; A15G; I18L; A20G; A21G; T25S; S26T; L27I; L28I; S29T; A30G; T181S; L182I; A183G; A185G; A186G; G187A; T188S; L190I; G192A; L194I; I199L; A201G; S204T; A208G; I210L; A385G; A388G; A389G; G390A; A392G; G393A; A395G; A396G; S399T; A400G; I401L; G403A; G404A; L407I; and A413G.

In a further embodiment, the present invention contemplates variants of the *R. sphaeroides* BtaA peptide defined by the amino acid sequence of SEQ ID NO: 3, wherein said variant comprises an amino acid substitution selected from the group consisting of:

H8K; H8R; R16H; R16K; H23K; H23R; R24H; R24K; R184H; R184K; R191H; R191K; R203H; R203K; H209K; H209R; R384H; R384K; R387H; R387K; H394K; H394R; R398H; R398K; H406K; H406R; R409H; R409K; R410H; R410K; R411H; and R411K.

In another embodiment, the present invention contemplates variants of the *R. sphaeroides* BtaB peptide defined by the amino acid sequence of SEQ ID NO: 4, wherein said variant comprises an amino acid substitution selected from the group consisting of:

T2S; A4G; T5S; A7G; A8G; L9I; A12G; T13S; I20L; A103G; L104I; L106I; G107A; T108S; I115L; L117I; S118T; A120G; L121I; S122T; G191A; A193G; S197T; L198I; G199A; G200A; G201A; A203G; I204L; L205I; G206A; T207S; L208I; and T209S.

In a further embodiment, the present invention contemplates variants of the *R. sphaeroides* BtaB peptide defined by the amino acid sequence of SEQ ID NO: 4, wherein said variant comprises an amino acid substitution selected from the group consisting of:

H6K; H6R; R15H; R15K; H16K; H16R; R18H; R18K; R19H; R19K; R111H; R111K; R114H; R114K; R196H; R196K; R210H; and R210K.

In one embodiment, the present invention contemplates a method for producing betaine lipids comprising: a) providing: i) a vector comprising DNA having an oligonucleotide

sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof; ii) a host cell; and b) transfecting said host cell with said vector. In a preferred embodiment, said host cell is a plant cell and said transfecting is performed under conditions such that the amount of phosphate fertilizer needed for the growth of crop plants is reduced.

5 It is not intended that the methods of the present invention be limited to any specific host cell capable of expressing the gene products encoded by isolated and purified DNA having an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. In one embodiment, said host cell is prokaryotic (*e.g. E. coli*). In another embodiment, said host cell is a eukaryotic (*e.g. yeast*). In another embodiment, said 10 host cell is a plant cell (*e.g. Arabidopsis, Maize, Soybean, Sorghum, Brassica, Medicago, Capsicum, Nicotiana, Zea, Triticum, and Datura*).

It is not intended that the methods of the present invention be limited to any specific vector. In one embodiment, said vector is selected from the group comprising pQE-31, pACYC-31, pBlueScript II SK+, pPCR-Script Amp, and pYES2.

15 In one embodiment, the present invention contemplates a method for producing betaine lipids *in vitro* comprising: a) providing: i) a first vector comprising DNA having the oligonucleotide sequence of SEQ ID NO: 1, and portions thereof ; ii) a second vector comprising DNA having the oligonucleotide sequence of SEQ ID NO: 2; iii) host cells; iv) *S*-adenosylmethionine; and v) diacylglycerol; b) transfecting said host cells with said first and 20 second vectors such that the gene products of said vectors are produced; and c) combining said gene products with said *S*-adenosylmethionine and said diacylglycerol *in vitro* under conditions such that betaine lipids are produced. In one embodiment, said host cells are selected from the group consisting of *E. coli*, *R. sphaeroides*, *M. loti*, and yeast. In a preferred embodiment, said host cells are plant cells.

25 It is not intended that the present invention be limited by the use of any specific method to express or produce betaine lipids including, but not limited to, DGTS. In one embodiment, the present invention contemplates the cloning of the *btaA* gene (SEQ ID NO: 1) into a protein expression vector selected from the group comprising pQE-9, pQE-16, pQE-30, pQE-31, pQE-32, pQE-40, pQE-60, pQE-70, pQE-80, pQE-81, pQE-82, pQE-100, pACYC-31, pBlueScript II 30 SK+, pPCR-Script Amp, and pYES2. In another embodiment, the present invention contemplates the cloning of the *btaB* gene (SEQ ID NO: 2) into said protein expression vectors.

In an alternative embodiment, the invention contemplates the transformation of plant cells or tissues such that the gene product encoded by the oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof, is expressed. In one embodiment, the present invention contemplates the cloning of the *btaA* gene (SEQ ID NO: 1) into a binary vector for introduction into *Agrobacterium tumefaciens*, and the subsequent generation of transgenic plant cells via Agrobacterial transformation. In another embodiment, the present invention contemplates the cloning of the *btaB* gene (SEQ ID NO: 2) into a binary vector for introduction into *Agrobacterium tumefaciens*, and the subsequent generation of transgenic plant cells via Agrobacterial transformation.

It is not intended that the invention be limited to the independent expression of the gene product encoded by the oligonucleotide sequence of SEQ ID NO:1, and portions thereof, in a single host cell, organism, or plant. Moreover, it is also not intended that the invention be limited to the independent expression of the gene product encoded by the oligonucleotide sequence of SEQ ID NO:2, and portions thereof, in a single host cell, organism, or plant. In one embodiment, the invention contemplates the co-expression of both of said gene products in a single host organism. In an alternative embodiment, the invention contemplates the transformation of plant cells or tissues such that both of said gene products are co-expressed.

It is not intended that the present invention be limited by the use of any specific method for the detection of betaine lipid production. The present invention contemplates a variety of assay formats. In one embodiment, a quantitative lipid assay utilizing thin layer chromatography (TLC) to detect the production of betaine lipids is contemplated. In another embodiment, an assay utilizing fast atom bombardment mass spectroscopy (FAB-MS) and proton-nuclear magnetic resonance (¹H-NMR) spectroscopy to measure the production of betaine lipids is contemplated.

In one embodiment, the production of the betaine lipid, DGTS, is visualized with iodine vapor and identified by co-chromatography with an *Arabidopsis thaliana* leaf lipid extract known to contain DGTS. In another embodiment, the production of DGTS is verified by quantitative analysis wherein reaction products are isolated from the TLC plates and used to prepare fatty acid methyl esters. The methyl esters are quantified by gas chromatography using myristic acid as the internal standard.

The methods of the present invention are conveniently carried out in a reaction vessel or container. It is not intended that the present invention be limited to any particular reaction vessel. A variety of containers can be used, including but not limited to, culture dishes, microwells, tubes, flasks and other glassware.

5 In addition, the present invention provides compositions comprising purified DNA having an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 44, and SEQ ID NO: 49. In some embodiments, RNA transcribed from the DNA is provided, while in other embodiments, protein translated from the RNA is provided. In further embodiments, antibodies produced from the protein are provided.

10 The present invention also provides vectors comprising DNA having an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 44, and SEQ ID NO: 49. In some embodiments, a host cell comprising the vector is provided. In some preferred embodiments, the host cell is selected from the group including but not limited to *E. coli*, *R. sphaeroides*, and *A. thaliana*. In further embodiments, transgenic plants comprising the vector are provided.

15 Moreover, the present invention provides compositions comprising isolated and purified DNA encoding a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 45, and SEQ ID NO: 50. In some embodiments, RNA transcribed from the DNA is provided, while in other embodiments, protein translated from the RNA is provided. In further embodiments, antibodies produced from the protein are provided.

20 The present invention also provides vectors comprising DNA encoding a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 45, and SEQ ID NO: 50. In some embodiments, a host cell comprising the vector is provided. In some preferred embodiments, the host cell is selected from the group including but not limited to *E. coli*, *R. sphaeroides*, and *A. thaliana*. In further embodiments, transgenic plants comprising the vector are provided.

25 Also provided by the present invention are purified nucleic acids that specifically hybridize to the complement of a sequence selected from the group consisting of SEQ ID NO:44, and SEQ ID NO:49, under highly stringent conditions in 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA at 68°C overnight, followed by washing in a solution comprising 0.1X SSPE and 0.1% SDS at 68°C, wherein the nucleic acid encodes a protein with DGTS synthetic activity. In some embodiments, vectors comprising the nucleic

acid, host cells comprising the vector or transgenic plants comprising the vector are provided. In some preferred embodiments, protein encoded by the nucleic acid is provided.

Additionally, the present invention provides purified nucleic acids comprising a sequence that is at least 95% identical to SEQ ID NO:44, or a sequence that is at least 95% identical to 5 SEQ ID NO:49, wherein the sequence encodes a protein with DGTS synthetic activity. In some embodiments, vectors comprising the nucleic acid, host cells comprising the vector or transgenic plants comprising the vector are provided. In some preferred embodiments, protein encoded by the nucleic acid is provided.

Moreover, the present invention provides peptides comprising the SAM binding domain 10 of *C. reinhardtii* Bta1, defined herein from residue 75 to 179 of SEQ ID NO:45. In further embodiments, the present invention provides peptides comprising the Bta domain, defined herein from residue 250 to 642 of SEQ ID NO:45. For instance, some embodiments comprise heterologous peptides attached to either the *C. reinhardtii* SAM binding domain or to the Bta 15 domain (e.g., fusion proteins). Suitable heterologous peptides include but are not limited to reporter sequences such as β-galactosidase, firefly luciferase and green fluorescent protein, and affinity tags such as polyhistidine, maltose binding protein and c-myc epitope.

Also provided are variant peptides, wherein the variant comprises an amino acid substitution including but not limited to: L77V; V85I; D86E; Y91F; I92V; D93N; L94V; A95S; K96E or S99T. In particularly preferred embodiments, the peptides are suitable for induction of 20 DGTS synthesis upon expression in *E. coli*.

The present invention further provides peptides comprising the SAM binding domain of *N. crassa* Bta1, defined herein from residue 184 to 254 of SEQ ID NO:50. In further embodiments, the present invention provides peptides comprising the Bta domain, defined herein from residue 495 to 812 of SEQ ID NO:50. For instance, some embodiments comprise 25 heterologous peptides attached to either the *N. crassa* SAM binding domain or to the Bta domain (e.g., fusion proteins). Suitable heterologous peptides include but are not limited to reporter sequences such as β-galactosidase, firefly luciferase and green fluorescent protein, and affinity tags such as polyhistidine, maltose binding protein and c-myc epitope.

Also provided are variant peptides, wherein the variant comprises an amino acid 30 substitution including but not limited to: Y497F; I498L; A500T; T502S; L512V; L513M;

N514E; L515I; A523T or I524L. In particularly preferred embodiments, the peptides are suitable for induction of DGTS synthesis upon expression in *E. coli*.

DESCRIPTION OF THE DRAWINGS

5 Figure 1 schematically shows the vector maps, including restriction endonuclease recognition sites, of the protein expression vectors pQE-30 (SEQ ID NO: 38), pQE-31 (SEQ ID NO: 39), and pQE-32 (SEQ ID NO: 40).

Figure 2 schematically shows the vector map, including restriction endonuclease recognition sites, of the phagemid vector pBlueScript II-SK(+).

10 Figure 3 schematically shows the proposed function of *btaA* and *btaB* in the biochemical pathway of DGTS biosynthesis in *R. sphaeroides*. DAG, diacylglyceryl; DGHS, diacylglycerylhomoserine; DGTS, diacylglyceryl-*N,N,N*-trimethylhomoserine; 5'-MTA, 5'-methylthioadenosine; SAM, *S*-adenosylmethionine; *S*-AHC, *S*-adenosyl homocysteine.

15 Figure 4 schematically shows the vector map, including restriction endonuclease recognition sites, of the protein expression vector pACYC184. This plasmid is a small, low copy-number *E. coli* cloning vector that is 4,244 base pairs in length and carries tetracycline (base numbers 1580-2770) and chloramphenicol-resistance (base numbers 219-3804) genes. The map shows the location of sites for restriction enzymes that cleave the molecule once or twice; unique sites are shown in bold type. The coordinates refer to the position 5' base in each
20 recognition sequence. Nucleotide number 1 of the vector is the first "G" of the unique *EcoRI* site, "GAATTC." The map also shows the relative positions of the antibiotic resistance genes and the origin of DNA replication (ORI) at base numbers 845-847. In order to generate the vector pACYC-31, a 459-bp *Xho* I-*Pvu* II fragment including the expression cassette from pQE-31 was isolated (See Figure 1) and ligated into the *Sal* I and *Eco* RV sites of pACYC184.

25 Figure 5 depicts a comparison of lipid extracts from different strains of *R. sphaeroides*. All cells were grown under phosphate-limited conditions at an initial P_i-concentration of 0.1 mM. A one-dimensional thin-layer chromatogram stained by iodine vapor is shown. The indicated strains and plasmids are described in Table 1. DGHS, diacylglycerylhomoserine; DGMS, diacylglyceryl-*N*-monomethyl homoserine; DGTS, diacylglyceryl-*N,N,N*-trimethylhomoserine.
30

Figure 6 depicts a two-dimensional thin layer chromatogram indicating the lipid phenotype of RKL3 containing *pptaA* (A) and a mutant disrupted in *btaB* (B). The cells were grown under phosphate-limited conditions at an initial P_i-concentration of 0.1 mM.

Abbreviations are as defined in the legend to Table 2.

5 Figure 7 shows the nucleic acid sequence of the *Rhodobacter sphaeroides btaA* gene (SEQ ID NO: 1) (submitted to GenBank data base and assigned accession number AF329857, nucleotide numbers 544-1790). The start and stop codons are highlighted and underlined, respectively, for emphasis.

10 Figure 8 shows the nucleic acid sequence of the *Rhodobacter sphaeroides btaB* gene (SEQ ID NO: 2) (submitted to GenBank data base and assigned accession number AF329857, nucleotide numbers 1791-2423). The start and stop codons are highlighted and underlined, respectively, for emphasis.

15 Figure 9 shows the amino acid sequence of the *Rhodobacter sphaeroides btaA* gene product (SEQ ID NO: 3) (submitted to GenBank data base and assigned accession number AF329857).

Figure 10 shows the amino acid sequence of the *Rhodobacter sphaeroides btaB* gene product (SEQ ID NO: 4) (submitted to GenBank data base and assigned accession number AF329857).

20 Figure 11 shows the structures of phosphatidylcholine (PC) and diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS). R1 and R2 represent the hydrocarbon chains' respective acyl groups.

Figure 12 schematically shows the vector map, including restriction endonuclease recognition sites, of the phagemid vector pPCR-Script Amp.

25 Figure 13 schematically shows the vector map, including restriction endonuclease recognition sites, of the yeast expression vector pYES2.

Figure 14 shows the nucleotide sequence of the mutagenesis oligonucleotide btaA-L9I (SEQ ID NO: 5). Said oligonucleotide correlates with base numbers 12-37 of SEQ ID NO: 1. The portion of the oligonucleotide wherein the mutation is encoded is indicated by a double-underline.

30 Figure 15 shows the nucleotide sequence of the mutagenesis oligonucleotide btaA-A201G (SEQ ID NO: 6). Said oligonucleotide correlates with base numbers 589-618 of SEQ ID

NO: 1. The portion of the oligonucleotide wherein the mutation is encoded is indicated by a double-underline.

Figure 16 shows the nucleotide sequence of the mutagenesis oligonucleotide btaA-S399T (SEQ ID NO: 7). Said oligonucleotide correlates with base numbers 1192-1217 of SEQ ID NO: 5 1. The portion of the oligonucleotide wherein the mutation is encoded is indicated by a double-underline.

Figure 17 shows the nucleotide sequence of the mutagenesis oligonucleotide btaB-T13S (SEQ ID NO: 8). Said oligonucleotide correlates with base numbers 24-51 of SEQ ID NO: 2. The portion of the oligonucleotide wherein the mutation is encoded is indicated by a double-
10 underline.

Figure 18 shows the nucleotide sequence of the mutagenesis oligonucleotide btaB-I115L (SEQ ID NO: 9). Said oligonucleotide correlates with base numbers 331-359 of SEQ ID NO: 2. The portion of the oligonucleotide wherein the mutation is encoded is indicated by a double-underline.

15 Figure 19 shows the nucleotide sequence of the mutagenesis oligonucleotide btaB-G206A (SEQ ID NO: 10). Said oligonucleotide correlates with base numbers 601-629 of SEQ ID NO: 2. The portion of the oligonucleotide wherein the mutation is encoded is indicated by a double-underline.

Figure 20 shows the results of an amino acid alignment and comparison of the *R. sphaeroides* btaA gene (SEQ ID NO: 3) and its *Mesorhizobium loti* gene homolog, Ml-btaA (SEQ ID NO: 41). Amino acid residues conserved between the two organisms are indicated by a black background. Amino acid residues which differ between the two organisms, but reflect a conservative amino acid change (e.g. leucine v. isoleucine) are indicated by a gray background.
20

Figure 21 shows the results of an amino acid alignment and comparison of the *R. sphaeroides* btaB gene (SEQ ID NO: 4) and its *Mesorhizobium loti* gene homolog, Ml-btaB (SEQ ID NO: 42). Amino acid residues conserved between the two organisms are indicated by a black background. Amino acid residues which differ between the two organisms, but reflect a conservative amino acid change (e.g. leucine v. isoleucine) are indicated by a gray background.
25

Figure 22 shows the nucleic acid sequence of the *R. sphaeroides* btaA gene homolog from *Mesorhizobium loti*, Ml-btaA (SEQ ID NO: 22) (GenBank accession number AP002997,
30

nucleotide numbers 269,421 to 270,667) as identified by BLAST search. The start and stop codons are highlighted and underlined, respectively, for emphasis.

Figure 23 shows the nucleic acid sequence of the *R. sphaeroides* *btaB* gene homolog from *Mesorhizobium loti*, Ml-*btaB* (SEQ ID NO: 23) (submitted to GenBank data base and assigned accession number AP002997, nucleotide numbers 270,670 to 271,347) as identified by BLAST search. The start and stop codons are highlighted and underlined, respectively, for emphasis.

Figure 24 shows the nucleic acid sequence of the *R. sphaeroides* *btaA* gene homolog from *Agrobacterium tumefaciens*, *btaA* (SEQ ID NO: 28).

Figure 25 shows the amino acid sequence of the *R. sphaeroides* *btaA* gene homolog from *Agrobacterium tumefaciens*, *btaA* (SEQ ID NO: 29).

Figure 26 shows the nucleic acid sequence of the *R. sphaeroides* *btaB* gene homolog from *Agrobacterium tumefaciens*, *btaB* (SEQ ID NO: 30).

Figure 27 shows the amino acid sequence of the *R. sphaeroides* *btaB* gene homolog from *Agrobacterium tumefaciens*, *btaB* (SEQ ID NO: 31).

Figure 28 shows the nucleic acid sequence of the *R. sphaeroides* *btaA* gene homolog from *Sinorhizobium meliloti*, *btaA* (SEQ ID NO: 32).

Figure 29 shows the amino acid sequence of the *R. sphaeroides* *btaA* gene homolog from *Sinorhizobium meliloti*, *btaA* (SEQ ID NO: 33).

Figure 30 shows the nucleic acid sequence of the *R. sphaeroides* *btaB* gene homolog from *Sinorhizobium meliloti*, *btaB* (SEQ ID NO: 34).

Figure 31 shows the amino acid sequence of the *R. sphaeroides* *btaB* gene homolog from *Sinorhizobium meliloti*, *btaB* (SEQ ID NO: 35).

Figure 32 provides a thin layer chromatogram of lipids extracted from *Chlamydomonas*, and *E. coli* transformed with an empty vector or a *Crpta1* expression vector.

Figure 33 provides an image of a nuclear magnetic resonance (NMR) analysis of DGTS purified from recombinant *E. coli* expressing *Crpta1*. The arrow is indicative of the quaternary ammonium function of DGTS.

Figure 34 provides two-dimensional thin layer chromatograms of lipids extracted from *N. crassa* under (A) high phosphate, and (B) phosphate-limited conditions, respectively.

Abbreviations are as follows: DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; PA,

phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; and PS, phosphatidyl serine.

Table 1. Description of Strains and Plasmids

Strain or Plasmid	Description or Construction
<i>R. sp.</i> 2.4.1.	wild type (ATCC #17023)
<i>R. sp.</i> RKL3	DGTS-deficient <i>btaA</i> MNNG induced mutant
<i>R. sp.</i> <i>btaB</i> -dis	DGTS-deficient <i>btaB</i> disruption mutant
<i>E. coli</i> HB101	F' $\Delta(mcrC-mrr)$ <i>leu supE44 ara14 galK2 lacY proA2 rpsL20 (Str^r) <i>xyl-5 mtl-l recA13</i></i>
<i>E. coli</i> XL-10 Gold	Tet ^r $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac$ Hte[F' <i>proAB lacIq ZDM15 Tn10</i> (Tet ^r) Amy Cam ^r] (Stratagene)
<i>E. coli</i> DH10B	F' <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80dlacZDM15 \Delta lacX74 deoR recA1 endA1 araD139 \Delta(ara, leu)7697 galU galKl- rpsL nupG$ (Gibco BRL)
<i>E. coli</i> MM294	F' <i>endA1 hsdR17 (r_k m_k) supE44 thi-1 relA1</i>
pBluescript II SK(+)	Amp ^r (Stratagene)
pRK2013	Kan ^r Tra ⁺ RK2-ColE1 _{rep}
pCHB500	Tc ^r ; expression vector for <i>R. sphaeroides</i>
pUC4K	Kan ^r Nm ^r ; contains neomycin phosphotransferase gene of Tn903 (Pharmacia)
pSUP202	Amp ^r Cm ^r Tc ^r pBR325 _{rep}
pRKL301	cosmid clone complementing RKL3
pRKL323	smallest subclone of pRKL301 complementing RKL3
<i>pbtAA</i>	nucleotides 436-1834 of pRKL323 in pCHB500
<i>pbtANT</i>	nucleotides 633-1834 of pRKL323 in pCHB500
<i>pbtAB</i>	nucleotides 1814-2625 of pRKL323 in pBS II SK(+)
<i>pbtAB-dis</i>	<i>btaB</i> inactivation cassette in pSUP202

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

"Analog" or "Analogs," as used herein, refers to polypeptides which are comprised of a segment of at least 25 amino acids that has partial identity (*i.e.* comprises an amino acid sequence of greater than 50%, and more preferably 70%, homology) to a portion of the deduced amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4, and which has (ideally) one or more properties of a transferase. The present invention contemplates utilizing the polypeptide transferases *S*-adenosyl methionine:diacylglycerol-3-amino-3-carboxyl transferase and *S*-adenosylmethionine: diacylglycerol homoserine-*N*-methyltransferase, to catalyze the formation of a detectable betaine lipid. The present invention also contemplates utilizing amino acid analogs (*e.g.* from *Mesorhizobium loti*, *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*) of the polypeptides encoded by SEQ ID NO: 1 and SEQ ID NO: 2.

"Associated peptide" as used herein refers to peptides that are bound directly or indirectly to other peptides. Associated peptides that are bound indirectly may have one or more peptides, or other molecules, bound between the two associated peptides. Peptides may be bound via peptide bonds, covalent bonds and non-covalent bonds. Peptides which co-precipitate are considered to be "associated peptides." For example, the present invention contemplates the co-precipitation of a polypeptide encoded by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and peptides associated thereto.

"Expression construct," "expression vector" and "plasmid" as used herein, refer to one or more recombinant DNA or RNA sequences containing a desired coding sequence operably linked to sequences necessary for the expression of the coding sequence in a cell or host organism (*e.g.*, mammal or plant). The sequence may be single or double stranded. The term "operably linked" refers to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced. The present invention contemplates expression vectors comprising an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 22, and SEQ ID NO: 23 (as well as homolog sequences described above).

"Reporter construct," "reporter gene" and "reporter protein" as used herein, refer to DNA or amino acid sequences, as appropriate, that, when expressed in a host cell or organism, may be detected, measured or quantitated. The present invention contemplates vectors further comprising reporter genes for easier detection of expression.

5 As used herein, the term "purified" or "to purify" refers to the removal of one or more (undesired) components from a sample. For example, where recombinant polypeptides are expressed in bacterial host cells, the polypeptides are purified by the removal of one or more host cell proteins, thereby increasing the percent of recombinant polypeptides in the sample. The present invention contemplates the purification of the polypeptides defined by SEQ ID NO: 10 3 and SEQ ID NO: 4 (and analogs thereof) by Ni-NTA/6xHis affinity column purification. The term purified encompasses a large range of purities such as "partially purified," "substantially purified," and to near homogeneity.

15 As used herein, the term "partially purified" refers to the removal of contaminants of a sample to the extent that the substance of interest is recognizable by techniques known to those skilled in the art (*e.g.*, by staining, blotting, etc.) as accounting for a measurable amount (*e.g.*, picograms, nanograms, micrograms, etc.) in the mixture. The present invention is not limited to compositions that are completely purified; in some embodiments, partially purified peptides are sufficient.

20 As used herein, the term "substantially purified" refers to molecules, (*e.g.*, nucleic or amino acid sequences) that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free and more preferably 90% free from other components with which they are naturally associated. The present invention is not limited to compositions that are substantially purified.

25 As used herein, when a solution passes through the solid support matrix, it comprises the "flow through." Material that does not bind, if present, passes with the solution through the matrix into the flow through. To eliminate all non-specific binding, the matrix is "washed" with one or more wash solutions which, after passing through the matrix, comprise one or more "effluents." "Eluent" is a chemical solution capable of dissociating material bound to the matrix (if any); this dissociated material passes through the matrix and comprises an "eluate." The 30 present invention contemplates the purification of antibodies by immobilizing peptides having

the amino acid sequence of SEQ ID NO: 3 and SEQ ID NO: 4 (and/or analogs thereof) on a support matrix.

"Antibody" as used herein, refers to a glycoprotein produced by B cells and plasma cells that binds with high specificity to an antigen (usually, but not always, a peptide) or a structurally similar antigen, that generated its production. Antibodies may be produced by any of the known methodologies and may be either polyclonal or monoclonal. An antibody demonstrates specificity to the immunogen, or, more specifically, to one or more epitopes contained in the immunogen.

"Staining," as used herein, refers to any number of processes known to those in the field (typically utilizing dyes) that are used to visualize a specific component(s) and/or feature(s) of a cell or cells. For example, the present invention contemplates quantitative lipid analysis wherein lipids are stained and visualized by exposure to iodine vapor and charring.

"Nucleic acid sequence," "nucleotide sequence," and "polynucleotide sequence" as used herein refer to an oligonucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single-, or double-stranded, and represent the sense or antisense strand.

As used herein, the terms "oligonucleotides" and "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 200 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a primer, probe, or amplimer.

The term "nucleotide sequence of interest" refers to any nucleotide sequence, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. Such nucleotide sequences include, but are not limited to, coding sequences of structural genes (*e.g.*, enzyme-encoding genes, transferase-encoding genes, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and of non-coding regulatory sequences that do not encode an mRNA or protein product (*e.g.*, promoter sequence, enhancer sequence, polyadenylation sequence, termination sequence, *etc.*).

"Amino acid sequence," "polypeptide sequence," "peptide sequence," and "peptide" are used interchangeably herein to refer to a sequence of amino acids.

The term "portion" when used in reference to a nucleotide sequence refers to fragments of that nucleotide sequence. The fragments may range in size from 5 nucleotide residues to the

entire nucleotide sequence minus one nucleic acid residue. The term "portion" when used in reference to an amino acid sequence refers to fragments of the amino acid sequence. The fragments may range in size from 3 amino acids to the entire amino acid sequence minus one amino acid residue. The present invention contemplates compositions comprising portions of the 5 oligonucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 2 (or homologs thereof). In some embodiments, the portions comprise at least 50%, preferably at least 75%, more preferably at least 90%, and most preferably at least 95% of a DNA sequence selected from the group including but not limited to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO 32, SEQ ID NO: 34, SEQ ID NO: 44, and SEQ 10 ID NO: 50. In other embodiments, the portions comprise at least 50%, preferably at least 75%, more preferably at least 90%, and most preferably at least 95% of an amino acid sequence selected from the group including but not limited to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO 33, SEQ ID NO: 35, SEQ ID NO: 45, and SEQ ID NO: 50. Particularly preferred embodiments, comprise portions of a BtaA, 15 BtaB or Bta1 amino acid sequence (or DNA encoding said amino acid sequence) which are "biologically active."

As used herein the term "SAM binding domain" refers to an amino acid sequence which comprises a SAM binding motif (sequences shared by proteins which bind S-adenosyl-L-methionine).

20 The term "Bta domain" as used herein, refers to an amino acid sequence which comprises a Bta (*e.g.*, BtaA, BtaB, Bta1, etc.) motif (sequences shared by proteins which function as S-adenosylmethionine:diacylglycerol 3-amino-3-carboxypropyl transferases).

An oligonucleotide sequence which is a "homolog" or a "variant" of a first nucleotide sequence is defined herein as an oligonucleotide sequence which exhibits greater than or equal to 25 50% identity, and more preferably greater than or equal to 70% identity, to the first nucleotide sequence when sequences having a length of 100 bp or larger are compared. The present invention contemplates compositions comprising homologs of the oligonucleotide sequence of SEQ ID NO:1 and SEQ ID NO: 2, and portions thereof. In some embodiments, the variants comprise at least 50%, preferably at least 75%, more preferably at least 90%, and most 30 preferably at least 95% identity to a DNA sequence selected from the group including but not limited to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 28,

SEQ ID NO: 30, SEQ ID NO 32, SEQ ID NO: 34, SEQ ID NO: 44, and SEQ ID NO: 49. In other embodiments, the variants comprise at least 50%, preferably at least 75%, more preferably at least 90%, and most preferably at least 95% identity to an amino acid sequence selected from the group including but not limited to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO 33, SEQ ID NO: 35, SEQ ID NO: 45, and SEQ ID NO:50. Particularly preferred embodiments, comprise variants of a BtaA, BtaB or Bta1 amino acid sequence (or DNA encoding said amino acid sequence) which are "biologically active."

As used herein, the term "biologically active" refers to a molecule having structural, regulatory and or biochemical functions of a wild type BtaA, BtaB or Bta1 molecule. In some instances, the biologically active molecule is a variant of a BtaA, BtaB or Bta1 molecule, while in other instance the biologically active molecule is a portion of a BtaA, BtaB or Bta1 molecule. Other biologically active molecules which find use in the compositions and methods of the present invention include but are not limited to mutant (*e.g.*, variants with at least one deletion, insertion or substitution) BtaA, BtaB or Bta1 molecules. Biological activity is determined for example, by restoration or introduction of Bta (*e.g.*, BtaA, BtaB or Bta1) activity in cells which lack Bta activity, through transfection of the cells with a *bta* expression vector containing a *bta* gene, derivative thereof, or portion thereof. Methods useful for assessing bta activity include but are not limited to reconstitution of the betaine lipid biosynthetic pathway as described in detail below in Example 2 (*e.g.*, restoration of DGTS synthesis).

The term "DGTS synthetic activity" as used herein, refers to the enzymatic activity or activities required for the production of DGTS (diacylglycerol-*N,N,N*-trimethylhomoserine) from SAM (S-adenosylmethionine) and DAG (diacylglycerol). The term DGTS synthetic activity includes either or both of 1) the production of DGHS from SAM and DAG, and 2) the production of DGTS from SAM and DGHS. In some embodiments, DGTS synthetic activity is assessed by transfection of a BtaA, BtaB or Bta1 expression vector into a host cell of interest, which is cultured under phosphate limited conditions. The transfected host cells are then harvested, and lipids are extracted and resolved by thin layer chromatography.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester

linkage. Therefore, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is referred to as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of another mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a 5 larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects that transcription proceeds in a 5' to 3' direction along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can 10 exert their effect even when located 3' of the promoter element and the coding region.

Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The term "cloning" as used herein, refers to the process of isolating a nucleotide sequence from a nucleotide library, cell or organism for replication by recombinant techniques.

15 The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to 20 nucleic acid molecules that transfer DNA segment(s) from one cell to another.

As used herein, the terms "complementary" or "complementarity" are used in reference to "polynucleotides" and "oligonucleotides" (which are interchangeable terms that refer to a sequence of nucleotides) related by the base-pairing rules. For example, the sequence "5'-CAGT-3'," is complementary to the sequence "5'-ACTG-3'." Complementarity can be "partial" 25 or "total." "Partial" complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules. "Total" or "complete" complementarity between nucleic acids is where each and every nucleic acid base is matched with another base under the base pairing rules. The degree of complementarity between nucleic acid strands may have significant effects on the efficiency and strength of hybridization between nucleic acid strands. This may 30 be of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids. The present invention contemplates the

hybridization of nucleic acids to the oligonucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2, under high stringency conditions.

The terms "homology" and "homologous" as used herein in reference to nucleotide sequences refer to a degree of complementarity with other nucleotide sequences. There may be 5 partial homology or complete homology (*i.e.*, identity). A nucleotide sequence which is partially complementary (*i.e.*, "substantially homologous") to a nucleic acid sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid sequence. The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or 10 Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous sequence to a target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one 15 another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target. The present invention contemplates the hybridization of nucleic acids homologous to the oligonucleotide sequences of 20 SEQ ID NO: 1 and SEQ ID NO: 2, to said sequences, under high stringency conditions.

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. "Stringency" typically occurs in a range from about T_m °C to about 20°C to 25°C below T_m . As will be understood by those of skill in the 25 art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under "stringent conditions" the nucleotide sequence of SEQ ID NO:1 and SEQ ID NO:2, or portions thereof, will hybridize to its exact complement and closely related sequences.

Low stringency conditions comprise conditions equivalent to binding or hybridization at 30 68°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄•H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent (50X Denhardt's

contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)) and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 2.0X SSPE, 0.1% SDS at room temperature when a probe of about 100 to about 1000 nucleotides in length is employed.

5 It is well known in the art that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, *etc.*) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol), as well as components 10 of the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, conditions which promote hybridization under conditions of high stringency (*e.g.*, increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, *etc.*) are well known in the art. High stringency conditions, when used in reference to 15 nucleic acid hybridization, comprise conditions equivalent to binding or hybridization at 68°C overnight, in a solution consisting of 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE and 0.1% SDS at 68°C, when a probe of about 100 to about 1000 nucleotides in length is employed. Other suitable highly stringent conditions include but are not limited to hybridization in 0.5 M 20 NaHPO₄, pH 7.2, containing 1% BSA, 5% SDS at 65°C overnight, followed by washing twice with 40 mM NaHPO₄, pH 7.2, containing 1% SDS, at 65°C.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize either partially or completely to either or both strands of the double-stranded nucleic acid 25 sequence under conditions of low stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe which can hybridize to the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "variant" or "variants" refers to analogs of the naturally 30 occurring *R. sphaeroides* proteins *S*-adenosylmethionine:diacylglycerol 3-amino-3-carboxyl transferase and *S*-adenosylmethionine:diacylglycerolhomoserine-*N*-methyltransferase that differ

in amino acid sequence or in ways that do not involve sequence, or both. Non-sequence modifications include *in vivo* or *in vitro* chemical derivatization of *S*-adenosyl methionine:diacylglycerol 3-amino-3-carboxyl transferase and *S*-adenosylmethionine:diacylglycerol homoserine-*N*-methyltransferase. Non-sequence modifications also include

5 changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation. Preferred variants include *S*-adenosyl methionine:diacylglycerol 3-amino-3-carboxyl transferase and *S*-adenosylmethionine: diacylglycerolhomoserine-*N*-methyltransferase (or biologically active fragments thereof) whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid

10 substitutions, deletions, or insertions which do not abolish the biological activity of *S*-adenosylmethionine:diacylglycerol 3-amino-3-carboxyl transferase and *S*-adenosylmethionine: diacylglycerolhomoserine-*N*-methyltransferase.

Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, *e.g.*, substitutions within the following groups: (Group I) acidic ((D) aspartate, (E) glutamate); (Group II) basic ((K) lysine, (R) arginine, (H) histidine); (Group III) nonpolar ((A) alanine, (V) valine, (L) leucine, (I) isoleucine, (P) proline, (F) phenylalanine, (M) methionine, (W) tryptophan); and (Group IV) uncharged polar ((G) glycine, (N) asparagine, (Q) glutamine, (C) cysteine, (S) serine, (T) threonine, (Y) tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. Conservative amino acid substitutions as contemplated by the present invention are presented in the following formula well-known in the field of art: "X₁ZX₂," wherein X₁ is the single-letter code for the amino acid residue present in the wild-type amino acid sequence (as indicated in SEQ ID NOS: 3 & 4), Z is the number of the amino acid residue being changed as a reflection of the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4, and X₂ is the single-letter code of the amino acid residue to which X₁ is being changed (*e.g.* I141L would indicate the changing of the isoleucine residue, at amino acid position 141, to a leucine residue). The present invention contemplates variants of the peptides encoded by SEQ ID NO: 3 and SEQ ID NO: 4 comprising a conservative amino acid substitution.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids using any process by which a strand of nucleic acid joins with a complementary strand through base pairing to form a hybridization complex. Hybridization and

the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids. The present invention contemplates the hybridization of nucleic acids and proteins to the 5 oligonucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

As used herein the term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bounds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic 10 acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (*e.g.*, C_{0t} or R_{0t} analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized to a solid support (*e.g.*, a nylon membrane or a nitrocellulose filter as employed in Southern and Northern blotting, dot blotting or a glass slide as employed in *in situ* hybridization, including FISH (fluorescent *in situ* 15 hybridization)).

As used herein, the term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of 20 the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.*, Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations which take structural as well as sequence characteristics into account 25 for the calculation of T_m . The present invention contemplates the hybridization of nucleic acids comprising the oligonucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof, at the T_m and above.

"Amplification" is defined herein as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art (*see, e.g.*, Dieffenbach and Dveksler, *PCR Primer, a Laboratory Manual*, Cold Spring 30 Harbor Press, Plainview NY [1995]). As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods of U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, all of

which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence (*e.g.* in a mixture of genomic DNA) without cloning or purification. The length of the amplified segment of the desired target sequence is determined by the relative positions of two oligonucleotide primers with respect to each other,
5 and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic
10 DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ^{32}P -labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments
15 created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications. The present invention contemplates the amplification of nucleic acid comprising SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof. The present invention contemplates the amplification of nucleic acids which are homologous to SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

20 The terms "reverse transcription polymerase chain reaction" and "RT-PCR" refer to a method for reverse transcription of a RNA sequence to generate a mixture of cDNA sequences, followed by increasing the concentration of a desired segment of the transcribed cDNA sequences in the mixture without cloning or purification. Typically, RNA is reverse transcribed using a single primer (*e.g.*, an oligo-dT primer) prior to PCR amplification of the desired
25 segment of the transcribed DNA using two primers.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the
30 presence of nucleotides and of an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in

amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the
5 primers will depend on many factors, including temperature, source of primer and the use of the method. The present invention contemplates portions of the oligonucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2 as useful for primers in DNA sequencing and PCR.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced
10 synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that it is detectable in any detection system, including, but not limited to enzyme
15 (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label. The present invention contemplates portions of the oligonucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2 as useful for probes in hybridization analysis (*e.g.* colony hybridization screening, Northern Blot, etc.) and PCR.

20 As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double- or single-stranded DNA at or near a specific nucleotide sequence. Restriction maps for the various vectors contemplated by the present invention are found in Figures 1, 2, 4, 12, and 13.

As used herein, the term "an oligonucleotide having a nucleotide sequence encoding a
25 gene" means a nucleic acid sequence comprising the coding region of a gene, *i.e.* the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers, promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close
30 proximity (*i.e.* operably linked) to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript.

Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers, splice junctions, intervening sequences, polyadenylation signals, etc. or a combination of both endogenous and exogenous control elements.

The term "promoter," "promoter element," or "promoter sequence" as used herein, refers 5 to a DNA sequence which when placed at the 5' end of (*i.e.*, precedes) an oligonucleotide sequence is capable of controlling the transcription of the oligonucleotide sequence into mRNA. A promoter is typically located 5' (*i.e.*, upstream) of an oligonucleotide sequence whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and for initiation of transcription.

As used herein, the terms "nucleic acid molecule encoding," "nucleotide encoding," 10 "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

The term "host cell" or "cell," as used herein, refers to any cell which is used to express a 15 "gene of interest," *e.g.* *btaA* and *btaB*. "Host cell" or "cell" also refers to any cell which is used in any of the screening assays for detection of the production of betaine lipids. The present invention contemplates host cells (*e.g.* bacteria, yeast, and plants) comprising the oligonucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated 20 oligonucleotide" refers to a nucleic acid sequence that is separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is nucleic acid present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA which are found 25 in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs which encode a multitude of proteins. The isolated nucleic acid or oligonucleotide may be present in single-stranded or double-stranded form. Isolated nucleic 30 acid can be readily identified (if desired) by a variety of techniques (*e.g.*, hybridization, dot blotting, etc.). When an isolated nucleic acid or oligonucleotide is to be utilized to express a

protein, the oligonucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide may be double-stranded).

As used herein the term "coding region" when used in reference to a structural gene
5 refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" (or "GTG" in some organisms) which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

10 As used herein, the term "gene" means the deoxyribonucleotide sequences comprising the coding region of a structural gene. A "gene" may also include non-translated sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The
15 sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into
20 heterogenous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In addition to containing introns, genomic forms of a gene may also include sequences
25 located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3' flanking region may contain sequences which
30 direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "transgenic" when used in reference to a cell refers to a cell which contains a transgene, or whose genome has been altered by the introduction of a transgene. The term "transgenic" when used in reference to a tissue or to a plant refers to a tissue or plant, respectively, which comprises one or more cells that contain a transgene, or whose genome has

5 been altered by the introduction of a transgene. Transgenic cells, tissues and plants may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into a target cell or integration of the transgene into a chromosome of a target cell by way of human intervention, such as by the methods described herein. The present invention contemplates transgenic cells comprising the oligonucleotide sequence of SEQ ID

10 NO: 1 and SEQ ID NO: 2, and portions thereof.

The term "transgene" as used herein refers to any nucleic acid sequence which is introduced into the genome of a cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring sequence. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence which contains some modification (*e.g.* a conservative amino acid substitution).

15

20 Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) which is introduced into the genome of a cell by experimental manipulations and may include gene sequences found in that cell so long as the introduced gene contains some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring gene. For example, the introduction of a gene having an oligonucleotide sequence selected from the group of SEQ ID NO: 1 and SEQ ID NO: 2 into a plant is the introduction of a foreign gene

25

30 into a plant.

The term "transformation" as used herein refers to the introduction of a transgene into a cell. Transformation of a cell may be stable or transient. The term "transient transformation" or "transiently transformed" refers to the introduction of one or more transgenes into a cell in the absence of integration of the transgene into the host cell's genome. Transient transformation
5 may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the transgenes. Alternatively, transient transformation may be detected by detecting the activity of the protein (e.g., *S*-adenosylmethionine:diacylglycerol 3-amino-3-carboxyl transferase and *S*-adenosylmethionine:
10 diacylglycerolhomoserine-*N*-methyltransferase) encoded by the transgene (e.g., the *btaA* and *btaB* genes, respectively) as demonstrated herein [e.g., quantitative analysis of lipid extracts to detect the production of DGTS]. The term "transient transformant" refers to a cell which has transiently incorporated one or more transgenes. In contrast, the term "stable transformation" or "stably transformed" refers to the introduction and integration of one or more transgenes into the genome of a cell. Stable transformation of a cell may be detected by Southern blot hybridization
15 of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the transgenes. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify transgene sequences. The term "stable transformant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA. "Functionally stable transformants" refers to stable transformants that
20 continue to express their incorporated transgenes. The present invention contemplates both stable and transient transformants comprising the oligonucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof.

A "transformed cell" is a cell or cell line that has acquired the ability to grow in cell culture for many multiple generations, the ability to grow in soft agar and the ability to not have
25 cell growth inhibited by cell-to-cell contact. In this regard, "transformation" refers to the introduction of foreign genetic material into a cell or organism. Transformation may be accomplished by any method known which permits the successful introduction of nucleic acids into cells and which results in the expression of the introduced nucleic acid. "Transformation" methods include, but are not limited to, such methods as microinjection, electroporation, and
30 DNA particle "bombardment." Transformation may be accomplished through use of any expression vector. For example, the use of *Agrobacterium tumefaciens* to introduce foreign

nucleic acid (*e.g.* having the oligonucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof) into plant cells is contemplated. Additionally, transformation refers to cells that have been transformed naturally, usually through genetic mutation.

The term "*Agrobacterium*" refers to a soil-borne, Gram-negative, rod-shaped

5 phytopathogenic bacterium which causes crown gall. The term "*Agrobacterium*" includes, but is not limited to, the strains *Agrobacterium tumefaciens*, (which typically causes crown gall in infected plants), and *Agrobacterium rhizogens* (which causes hairy root disease in infected host plants).

The terms "bombarding," "bombardment," and "biolistic bombardment" refer to the

10 process of accelerating particles towards a target biological sample (*e.g.*, cell, tissue, *etc.*) to effect wounding of the cell membrane of a cell in the target biological sample and/or entry of the particles into the target biological sample. Methods for biolistic bombardment are known in the art (*e.g.*, U.S. Patent No. 5,584,807, the contents of which are herein incorporated by reference), and are commercially available (*e.g.*, the helium gas-driven microprojectile accelerator (PDS-

15 1000/He) (BioRad).

The term "microwounding" when made in reference to plant tissue refers to the introduction of microscopic wounds in that tissue. Microwounding may be achieved by, for example, particle bombardment as described herein.

The term "plant" as used herein refers to a plurality of plant cells which are largely

20 differentiated into a structure that is present at any stage of a plant's development. Such structures include, but are not limited to, a fruit, shoot, stem, leaf, flower petal, *etc.* The term "plant tissue" includes differentiated and undifferentiated tissues of plants including, but not limited to, roots, shoots, leaves, pollen, seeds, tumor tissue and various types of cells in culture (*e.g.*, single cells, protoplasts, embryos, callus, protocorm-like bodies, *etc.*). Plant tissue may be 25 *in planta*, in organ culture, tissue culture, or cell culture.

The term "embryonic cell" as used herein in reference to a plant cell refers to one or more plant cells (whether differentiated or un-differentiated) which are capable of differentiation into a plant tissue or plant. Embryonic cells include, without limitation, protoplasts such as those derived from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, 30 *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*,

Ciohorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browalia, Glycine, Lolium, Zea, Triticum, Sorghum, and Datura. Also included are embryos (such as those from sorghum, maize, banana), embryonic meristems (such as those from soybean),

5 embryogenic callus (such as from sugarcane), protocorm-like bodies (such as from pineapple), and embryogenic cells as exemplified by those from garlic. The ability of an embryonic cell to differentiate into a plant is determined using methods known in the art. For example, differentiation of pineapple protocorm-like bodies into shoots may be accomplished by culturing the protocorm-like body on agar-solidified hormone-free modified Murashige & Skoog (MS) medium or on agar-solidified PM2 medium (U.S. Patent No. 6,091,003 hereby incorporated by reference). Differentiation into pineapple roots may be accomplished by culture of protocorm-like bodies in liquid modified MS medium containing 1 mg/L NAA.

10

15

The term "conjugation" as used herein refers to the process in which genetic material is transferred from one microorganism to another involving a physical connection or union between the two cells. This process is commonly known to occur in bacteria, protozoa, and certain algae and fungi.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for the production of betaine lipids. The compositions of the present invention comprise isolated and purified DNA having an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, and portions thereof (as well as the homologs described above, and portions thereof). The methods of the present invention comprise the expression of recombinant enzymes (e.g. from *Rhodobacter sphaeroides*) in host cells (e.g. in bacteria and plants) to produce betaine lipid compounds including, but not limited to, Diacylglyceryl-O-4'-(*N,N,N*-trimethyl) homoserine (DGTS). The compositions and methods of the present invention allow a reduction in the amount of plant cell membrane-associated phosphorus by replacing phosphorous lipids with non-phosphorous lipids. Thus, the overall amount of phosphate-containing fertilizer required for the growth of the plant is reduced.

20

25

Polar lipids are essential components of all biological membranes. Most common are glycerolipids containing a diacylglycerol moiety to which a polar head group is attached. A

head group can be a carbohydrate moiety as in the very abundant plant galactolipids or a phosphorylester as in the glycerophospholipids, the most common lipid class in animals. Betaine lipids represent a third class of glycerolipids in which a quaternary amine alcohol is bound in an ether linkage to the diacylglycerol moiety. Betaine lipids are structural components
5 of membranes in ferns, mosses, algae, and bacteria. The overall structure of betaine lipids resembles to some extent that of the glycerophospholipid phosphatidylcholine (PC). Although the phase transition temperature for betaine lipid is slightly higher compared to PC with identical fatty acid composition, the physical phase behavior of betaine lipid in mixtures with water is similar to that of PC.

10 The betaine lipid diacylglycerol-*N*-trimethylhomoserine (DGTS) is similar in structure to the common phosphoglycerolipid, phosphatidylglycerol (PC) (*See Figure 11*), but lacks phosphorous. PC plays an important and central role in lipid metabolism in seed plants. However, many organisms alter their lipid composition in response to chemical or physical changes of the environment, permitting the organism to survive unfavorable conditions. For
15 example, DGTS replaces PC to the extent that PC is actually absent in some algae. Thus, DGTS could take over these functions in organisms lacking PC.

Plants depend much less on phospholipids than animals. Recent discoveries indicate that plants are able to replace phospholipids with non-phosphorous glycolipids to conserve phosphate under phosphate-limiting growth conditions. Agricultural phosphate overfertilization
20 creates environmental problems and will lead to a depletion of naturally occurring phosphate fertilizer resources in the near future. Therefore, it is highly desirable to develop new strategies to reduce the amount of phosphate fertilizer needed for the optimal growth of crop plants. The invention illustrates such a strategy by providing compositions and methods that reduce the amount of plant cell membrane-associated phosphorus (which represents approximately 30% of
25 all organic phosphorus in a plant cell) by replacing the phosphorous lipids with non-phosphorous lipids.

The present invention is not limited by any specific reaction mechanism, and indeed it is not necessary to understand any particular underlying mechanism in order to practice the invention. It is believed that the production of the betaine lipid, DGTS, is driven by the activity
30 of the *R. sphaeroides btaA* gene product, *S*-adenosyl methionine:diacylglycerol 3-amino-3-

carboxyl transferase, coupled with the activity of the *R. sphaeroides* *btaA* gene product, S-adenosylmethionine:diacylglycerol homoserine-*N*-methyltransferase.

I. Cloning and Expression of the *R. sphaeroides* *btaA* and *btaB* genes in *E. coli*

The present invention provides methods for the production of betaine lipids, including but not limited to DGTS, wherein the *btaA* (SEQ ID NO: 1) and *btaB* (SEQ ID NO: 2) genes of *R. sphaeroides* are cloned into, and expressed in, *Escherichia coli* cells. Although the present invention is not limited to a specific method whereby said genes are cloned and expressed in *E. coli*, in one embodiment, said genes are cloned and expressed as follows.

A. Cloning

1. Growth of *R. sphaeroides* Cell Cultures

Although the present invention is not limited to a specific method of growing cell cultures of *R. sphaeroides*, in one embodiment, said cell cultures are grown, and genomic DNA is isolated and purified therefrom, as follows.

Cell cultures were grown in the malate-basal-salt medium as described by Ormerod *et al.*, "Light-dependent utilization of organic compounds and photoreduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism," *Arch. Biochem. Biophys.*, 94: 449-463 (1961), or Sistrom's succinate-basal-salt medium. (See Sistrom W.R., "A requirement of sodium in the growth of *R. sphaeroides*," *J. Gen. Microbiol.*, 22:778-785 (1960); Sistrom W.R., "The kinetics of the synthesis of photopigments in *R. sphaeroides*," *J. Gen. Microbiol.* 28: 607-616 (1962). Agar plates (1.5% agar) were either incubated in the dark at 30°C in air or in the light (100 μE m⁻² s⁻¹) at 30-35°C in an atmosphere containing 5% CO₂ and 95% N₂. When required, 0.8 μg/ml tetracycline was added to agar plates containing Sistrom's medium. Aerobic chemoheterotrophic, liquid cultures inoculated with a single colony were incubated at 30°C with shaking in Erlenmeyer flasks. Anaerobic photoheterotrophic, liquid cultures were grown in tightly-closed, filled, 200 ml bottles, in the light (100 μE m⁻² s⁻¹) at 30-35°C. The bottles are mixed for aeration once or twice a day by manual shaking.

2. Preparation of genomic DNA from *R. sphaeroides*

Although the present invention is not limited to a specific method of purifying nucleic acids from *R. sphaeroides*, in one embodiment, genomic DNA is isolated and purified from *R. sphaeroides* as follows. The DNA prepared by this method is suitable for endonuclease restriction, Southern Blotting, and PCR applications.

R. sphaeroides cells are grown as noted above and 3 ml of the bacterial culture is centrifuged at 10,000 x g in a 1.5 mL polypropylene tube. The bacterial cell pellet is resuspended in 1 ml TE buffer (Tris-Cl, 50 mM; EDTA, 1mM; pH 8.0). The cells are re-centrifuged, followed by resuspension of the pellet in 1 mL TE buffer containing 1% SDS, 0.5 mg/mL Proteinase K. The cells are incubated for 1 hour at 37°C. To shear the genomic DNA, the sample is extruded from a syringe through a G20-1.5 needle. The DNA preparation is sequentially extracted with an equal volume of phenol, phenol/chloroform (1:1, v/v), and chloroform/isoamylalcohol (24:1, v/v), a technique that is well known in the field of art. The DNA is precipitated by adding 0.3 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 200 proof ethanol to the extracted DNA. The DNA is pelleted by centrifugation at 15,000 x g in a microcentrifuge for 2 minutes. The DNA pellet is air-dried and resuspended in 0.1 mL TE-buffer.

3. PCR of *BtaA* and *BtaB* from genomic DNA and cloning of the PCR product into a Bacterial Expression Vector

In one embodiment, in order to clone the *btaA* and *btaB* genes of *R. sphaeroides* into *Escherichia coli*, *R. sphaeroides* genomic DNA (isolated and purified as indicated above) is subjected to Polymerase Chain Reaction (PCR) such that the genomic sequences for *btaA* and *btaB* are respectively generated.

In order to amplify the *btaA* gene, a forward primer having the nucleotide sequence 5'-ACA TGC ATG CAG TGA CGC AGT TCG CCC TC-3' (SEQ ID NO: 36), and a reverse primer having the nucleotide sequence 5'-CGG GGT ACC AGG ACG ATC CGC TCG AAC CG-3' (SEQ ID NO: 37), were used such that *Bam* H1 and *Hind* III sites were provided for cloning into the bacterial expression vector pPCR-Script Amp (Stratagene Cat. No. 211188), a derivative of pBlueScript SK+. The forward primer amplifies the beginning of the gene, including the *Val* start site (GTG) (See Figure 7: SEQ ID NO: 1). The reverse primer includes

the stop codon of the *btaA* gene in the resulting PCR product. PCR products are run on 1% Tris-Acetate-EDTA (TAE) agarose gel in the presence of ethidium bromide and excised for purification by QIAEX II gel extraction kit (Qiagen Cat. No. 20021), followed by cloning into the *Srf*I site of pPCR-Script Amp (as described in the manufacturer's instructions). The 5 resulting plasmid construct allows the expression of the recombinant *btaA* gene product in *E. coli*.

In order to amplify the *btaB* gene, a forward primer having the nucleotide sequence 5'-ACA TGC ATG CAG TGA CGC AGT TCG CCC TC-3' (SEQ ID NO: 36), and a reverse primer having the nucleotide sequence 5'-CGG GGT ACC AGG ACG ATC CGC TCG AAC 10 CG-3' (SEQ ID NO: 37), were used such that *Sph* I and *Kpn* I sites were provided for cloning into pPCR-Script Amp. The forward primer amplifies the beginning of the gene, including the *Met* start site (ATG) (See Figure 8: SEQ ID NO: 2). The reverse primer includes the stop codon 15 of the *btaB* gene in the resulting PCR product. PCR products are run on 1% TAE agarose gel in the presence of ethidium bromide and excised for purification by QIAEX II gel extraction kit followed by cloning into the *Srf*I site of pPCR-Script Amp (as described in the manufacturer's 20 instructions). The resulting plasmid construct allows the expression of the recombinant *btaB* gene product in *E. coli*.

In an alternative embodiment, the *btaA* and *btaB* genes are amplified as indicated above, but are cloned into a pBluescript II SK+ phagemid vector (Stratagene Cat. No. 212205). Both 25 the vector and the PCR products are digested with the appropriate restriction endonucleases (*i.e.* digested with *Bam* H1 and *Hind* III for *btaA*, or digested with *Sph* I and *Kpn* I for *btaB*) prior to ligation to allow direct cloning of the products into the vector.

B. Expression of the *btaA* and *btaB* Gene Products

The present invention is not limited to a specific method of expressing the *btaA* and *btaB* gene products from *R. sphaeroides* (*i.e.* the proteins encoded by the amino acid sequences found in SEQ ID NO: 3 and SEQ ID NO: 4, respectively). However, in one embodiment, the 30 invention contemplates the expression of the *R. sphaeroides* *btaA* and *btaB* gene in *E. coli*.

1. Cloning of *btaA* and *btaB* PCR inserts into *E. coli* expression vectors pQE-31 and pACYC-31

In one embodiment, *btaA* and *btaB* genes generated from PCR (as described above) are cloned into either the pQE-31 (Figure 1)(QIAGEN Cat.# 32915) or pACYC-31 vectors. The vector

5 pACYC-31 is described in the publication Dormann *et al.*, "Arabidopsis galactolipid biosynthesis and lipid trafficking mediated by DGD1," *Science*, 284: 2181-2184. (See Figure 4). Said vectors are digested with *Sph* I and *Kpn* I (for *btaA*) or *Bam* HI and *Hind* III (for *btaB*), and gel purified using the QIAEX II gel extraction kit (Qiagen Cat. No. 20021). The *btaA* PCR insert is excised from pBlueScript SK+ by *Sph* I/*Kpn* I digest and gel purified as above. The
10 *btaB* PCR insert is released from the pBlueScript SK+ vector by *Bam* HI/ *Hind* III digestion and is then gel purified. The inserts and vectors are ligated together with T4 DNA ligase, a technique well-known in the field of art. Ligation reactions are then transformed into electroporation-competent *E. coli* XL1-Blue (Stratagene Cat. No. 200228) and plated onto LB Ampicillin plates (*i.e.* for pQE-31) or LB Chloramphenicol plates (*i.e.* for pACYC-31).

15 Each construct is analyzed individually for protein expression (as detailed in the Qiagen QIAexpress System literature) using *E. coli* M15[pREP4] (QIAGEN Cat. No. 34210) as an expression host for the pQE-31 based plasmids and XL1-Blue as the host for pACYC-31 based constructs. Since the pACYC-31 and pQE-31 vectors carry compatible origins of replication, reconstitution of the DGTS biosynthetic pathway is achieved by concurrent expression of
20 pACYC-31:*BtaA* and pQE-31:*BtaB*, or pACYC-31:*BtaB* and pQE-31: *BtaA* in XL1-Blue. Transformed *E. coli* cells are grown and subsequently analyzed by TLC (as described below) for DGTS production after induction with 1-5 mM isopropyl- β -D-thiogalactoside (IPTG) (Amersham Pharmacia Biotech, Piscataway, NJ: Cat. No. 27-3054-03).

25 The present invention is not limited to the use of any specific protein expression vector or system. In one embodiment, the protein expression vector is selected from the group comprising pQE-9, pQE-16, pQE-30, pQE-31, pQE-32, pQE-40, pQE-60, pQE-70, pQE-80, pQE-81, pQE-82, pQE-100 (all available from QIAGEN, Inc., Valencia, CA). In another embodiment, the protein expression vector is selected from the group comprising pACYC-31 and pACYC184 (New England Biolabs, Beverly, MA: Cat. No. E4152S).

30 The present invention is not limited to any specific means of purifying recombinant *R. sphaeroides* *btaA* and *btaB* gene products. In one embodiment, the resulting plasmid constructs,

pQE-31: *BtaA* and pQE-31:*BtaB*, allowed the expression of their respective recombinant *R. sphaeroides* *bta* gene products in *E. coli*. Moreover, said plasmid constructs allow the purification of said gene products due to the selective binding of the six N-terminal histidine residues (*i.e.* 6xHis tag) of the plasmid construct to nickel nitriloacetic acid (Ni-NTA) agarose resin, following the manufacturer's instructions. (QIAGEN, Inc., Valencia, CA: Cat. No. 30210). The recombinant *R. sphaeroides* *bta* gene products are eluted with 200 mM imidazole (which was subsequently removed by use of a Millipore Ultrafree 4 concentrator (Millipore, Inc., Bedford, MA)) and stored in a buffer comprising glycerol, NaCl, and NaH₂PO₄ (pH 7.5) at -20°C.

10

2. Expression of *BtaA* and *BtaB* in Yeast using the pYES2 system

It is not intended that the present invention be limited solely to the expression of the *btaA* and *btaB* gene products from *R. sphaeroides* in *E. coli*. In one alternative embodiment, the present invention contemplates the expression of said gene products, resulting in the production of betaine lipids (including but not limited to DGTS) in yeast as follows.

In order to amplify the *btaB* gene from *R. sphaeroides* genomic DNA (isolation as described above), a forward primer having the nucleotide sequence 5'-GCA AAG CTT AGC ATG GCC GAC GCC ACC CAT-3' (SEQ ID NO: 8), and a reverse primer having the nucleotide sequence 5'-GCA GGA TCC CTC TCA CCG CGT GAG CGT G-3' (SEQ ID NO: 9), were used such that *Bam*H1 and *Hind* III sites were provided for cloning into the yeast expression vector pYES2 (Invitrogen Cat. No. V825-20).

In order to amplify the *btaA* gene from *R. sphaeroides* genomic DNA (isolation as described above), a forward primer having the nucleotide sequence 5'-CGG GGT ACC ATG GCG CAG TTC GCC CTC-3' (SEQ ID NO: 9), and a reverse primer having the nucleotide sequence 5'-ACA TGC ATG CAG GAC GAT CCG CTC GAA CCG-3' (SEQ ID NO: 10), were used such that *Sph* I and *Kpn* I sites were provided for cloning into pYES2.

The reaction mixtures and thermal cycling conditions are the same as those noted below in the Examples. PCR products are run on a 1% TAE agarose gel in the presence of ethidium bromide and excised for purification by QIAEX II gel extraction kit, followed by ligation into the appropriate restriction sites of pYES2 (*i.e.* *Sph* I and *Kpn* I sites or *Bam* H1 and *Hind* III sites). Ligation reactions are transformed into XL-1 Blue cells, and the resultant constructs

purified and transformed into INVSc1 *ura3* yeast cells (Invitrogen Cat. No. V825-20) as described in the pYES2 product literature. The resulting plasmid constructs allow the expression of the recombinant *btaA* and *btaB* gene products in yeast.

Yeast cells transformed by this method are grown and subsequently analysed by TLC (as 5 described below) for DGTS production after induction with 2% galactose-containing medium.

3. Co-Expression of *R. sphaeroides* *btaA* and *btaB* Gene Products

It is not intended that the invention be limited to the independent expression of a peptide having the amino acid sequence selected from the group of SEQ ID NO: 3 and SEQ ID NO: 4, 10 or portions thereof, in a single host organism or plant. In one embodiment, the invention contemplates the co-expression of both of the peptides described above in a single host organism or plant. In one embodiment, co-expression of the peptides encoded by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4 (for example, in separate protein expression vectors) in *E. coli*, such that a betaine lipid biosynthetic pathway 15 (e.g. produces DGTS) is reconstituted, is contemplated as follows.

In order to express two proteins in *E. coli*, two compatible plasmids with the ability to express proteins, one for *btaA* and one for *btaB*, are utilized. Each plasmid must have a different antibiotic resistance marker in order to select for transformants with the correct combination of plasmids. The plasmid pQE-31 provides ampicillin resistance, whereas the plasmid, pACYC- 20 31, provides chloramphenicol resistance. The *btaA* and *btaB* genes from *R. sphaeroides* are cloned into pQE-31 and pACYC-31 as described above. The M15 cell line (QIAGEN, Inc., Valencia, CA) is transformed with a pQE-31/*btaA* protein expression construct (as described above). The pACYC-31/*btaB* expression construct is transformed into the M15 cell line containing the pQE-31/*btaA* expression vector. Upon induction of expression with 1-5 mM 25 isopropyl- β -D-thiogalactoside (IPTG) (Amersham Pharmacia Biotech, Piscataway, NJ: Cat. No. 27-3054-03), both proteins are expressed.

C. Detection of Betaine Lipid Production

It is not intended that the present invention be limited by any specific means of detecting 30 the production of betaine lipids, including but not limited to DGTS, by the compositions and methods contemplated herein. In one embodiment, detection of the production of betaine lipids

comprises thin layer chromatography and visualization with iodine vapor. In another embodiment, detection of the production of betaine lipids comprises quantitative lipid analysis wherein reaction products are isolated from the TLC plates and used to prepare fatty acid methyl esters. The methyl esters are quantified by gas chromatography using myristic acid as the internal standard. In an alternative embodiment, detection of the production of betaine lipids comprises lipid extraction followed by fast atom bombardment-mass spectroscopy (FAB-MS).

1. Detection of DGTS Production by Thin Layer Chromatography (TLC)

Randomly chosen colonies from a population of *R. sphaeroides* cells known to produce the lipid, DGTS, are streaked as small patches (0.5 by 0.5 cm) on fresh Z-broth plates. Lipids are isolated from these patches by collecting cells onto the wide end of a flat toothpick and swirling the material in 75 µl of chloroform-methanol (1:1, vol/vol) contained in polypropylene microcentrifuge tubes. Following the addition of 25 µl of 1 N KCl-0.2 M H₃PO₄, the tubes are vortexed and centrifuged to separate the organic and aqueous phases. A 10 µl aliquot is withdrawn from the lipid-containing lower phase and directly spotted onto an activated ammonium sulfate-impregnated silica gel thin layer chromatography (TLC) plate for one-dimensional lipid separation. For this purpose, Baker Si250 silica plates with a pre-adsorbent layer are prepared by soaking in 0.15 M ammonium sulfate for 30 seconds, followed by air drying to complete dryness. Immediately prior to use, the plates are activated for 2.5 h at 120°C.

Activation of ammonium sulfate-treated plates at 120°C produces sulfuric acid, which protonates phosphatidylglycerol, making it less polar. An acetone-benzene-water mixture (91:30:8, vol/vol/vol) is employed as the solvent system. Lipids were visualized by spraying the plates with 50% sulfuric acid followed by heating at 160°C for 10 to 15 minutes to char the lipids. (See Figure 5).

25

2. Quantitative Lipid Analysis to Verify the Production of DGTS

It is not intended that the present invention be limited to any specific method of verifying the production of betaine lipids including, but not limited to, DGTS. In one embodiment, a method for quantitative lipid analysis of lipids produced by the present invention is contemplated as described in Benning C. & Somerville C.R., "Isolation and Genetic

Complementation of a Sulfolipid-Deficient Mutant of *Rhodobacter sphaeroides*," *J. Bacteriol.*, 174: 2352-2360 (1992).

For each strain, three 50-ml cultures were grown aerobically in Sistrom's medium (as described above) with shaking at 32°C in the dark. The cells were grown under phosphate-limited conditions at an initial P_i concentration of 0.1 mM. The cells were centrifuged, suspended in 0.5 ml of water, and extracted by vortexing with 4 ml of chloroform-methanol (1:1, vol/vol). Addition of 1.3 ml of 1 M KCl-0.2 M H₃P0₄, vortexing, and centrifugation resulted in phase partitioning of the lipids into the lower chloroform phase. The chloroform phase was removed and concentrated to 0.2 ml by evaporation under a stream of N₂. The sample was split, and the material was spotted onto activated (30 min at 110°C) silica TLC plates (Si250; Baker). The plates were developed in two dimensions, first with chloroform-methanol-water (65:25:4, vol/vol/vol), and then with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume).

Lipids were visualized with iodine vapor (*See Figure 6*), and after desorption of iodine, the spots were individually scraped into 8-ml screw-cap tubes. To the samples, 5 µg of myristic acid methyl ester in 0.1 ml of hexane was added as an internal standard, since only negligible amounts of endogenous myristic acid are found in the bacterial lipids. Fatty acid methyl esters were prepared by addition of 1 ml of anhydrous 1 N methanolic HCl (Supelco) followed by incubation at 80°C for 1 h. Following the addition of 1 ml of 0.95% (wt/vol) KCl, the fatty acid methyl esters were extracted into 1 ml of hexane and then dried to a volume of 0.1 ml.

Samples (2 µl each) were injected onto a gas chromatograph (Varian 2000) which was equipped with a 2.4-m column (2-mm inner diameter) packed with 3% SP-2310 and 2% SP-2300 on 100/120 Chromosorb WAW (Supelco). The carrier gas (N₂) flow rate was adjusted to 20 ml/min, and the column temperature was set for 2 min at 180°C, increasing to 200°C over 10 min, and 4 min at 200°C.

The fatty acid methyl esters were detected by a flame ionization detector, and the data were integrated by a Spectra Physics integrator. To calculate the relative amounts of the polar lipids included in the analysis, the amount of fatty acids contained in each lipid of a particular sample was calculated from the resulting gas chromatogram based on the following formula:

[(total area under all peaks - standard peak area)/standard peak area] X 5 µg. The relative amount for each lipid in the sample was expressed as a percent of all lipids analyzed (*See, Table*

2). The validity of calculation was based on the assumption that each of the lipids, including the unidentified lipids, contain two fatty acids per molecule, and that the different lipids have a similar fatty acid composition.

5

Table 2. Lipid Composition of *R. sphaeroides* Wild Type and RKL3 Following Phosphate Deprivation

Lipid	WT (mol%)		RKL3 (mol%)	
MHDG	1.3	± 0.2	12.7	± 1.6
DGTS	15.9	± 1.7	ND	
MPE	ND		0.9	± 0.2
GGDG	32.6	± 2.4	36.7	± 2.8
PE	1.5	± 0.1	7.5	± 0.4
OL	21.2	± 3.6	15.2	± 0.1
PG	4.7	± 0.6	7.5	± 0.4
SQDG	17.5	± 0.9	14.5	± 0.7
PC	0.1	± 0.1	1.1	± 0.6
PL	5.3	± 0.5	3.7	± 0.2

Mean values from three independent cultures (0.1 mM P_i) and standard errors are shown.

Abbreviations: DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; GGDG,

10 glucosylgalactosyldiacylglycerol; MHDG, monohexosyldiacylglycerol; MPE, N-monomethylphosphatidylethanolamine; ND, not detected; OL, ornithine lipid; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, undefined phospholipid; SQDG, sulfoquinovosyldiacylglycerol.

15

3. Confirmation of Betaine Lipid Production by FAB-MS and ¹H-Nuclear Magnetic Resonance

It is not intended that the present invention be limited any specific method of confirming betaine lipid production. In one embodiment, a method for confirming the production of the betaine lipid, DGTS, is contemplated comprising fast atom bombardment mass

spectroscopy (FAB-MS) and ^1H -NMR-spectroscopy as described in Benning *et al.*, "Accumulation of a Novel Glycolipid and a Betaine Lipid in Cells of *Rhodobacter sphaeroides* Grown Under Phosphate-Limitation," *Arch. Biochem. Biophys.*, 317: 103-111 (1995). Lipids produced by the present invention (*e.g.* DGTS) may be analyzed by FAB-MS and ^1H -NMR-spectroscopy and compared to the predicted values for a range of lipids, and more specifically, betaine lipids, in order to confirm production of the desired lipid.

5 FAB-MS measurements were done at the MSU-NIH Mass Spectrometry Facility using a JEOL HX-110 double focusing mass spectrometer (JEOL USA, Peabody, MA) operating in the negative ion mode (for glycolipids) or in the positive ion mode (for betaine lipids).

10 Approximately 1 μg of lipid was mixed with 1 μl of matrix; either triethanolamine for glycolipids, or glycerol/15-crown-5 (2:1, v/v) for the betaine lipid. Ions were produced by bombardment with a beam of Xe atoms (6 keV). The accelerating voltage was 10 kV and the resolution was set at 1000. Exact mass measurements were obtained by peak matching at a resolution of ca. 10,000 to either a matrix ion or an ion of a reference compound added to the sample.

15 Briefly, the fatty acid composition of the Dragendorff-positive lipid (*i.e.* DGTS) accumulating in phosphate-starved cells of *R. sphaeroides* was determined. With *cis*- Δ^{11} -octadecenoyl (vaccenoyl) at 87.5 mol% as the predominant fatty acid, simple patterns for mass- and ^1H -NMR-spectroscopy of purified DGTS samples containing a mixture of molecular species were expected. Positive mode FAB-MS indicated DGTS had molecular ion, $[\text{M}+\text{H}]^+$, 20 at m/z 764. By high resolution FAB-MS peak matching to a reference ion ($[\text{M}+\text{H}]^+$ at m/z 734.4691 for $\text{C}_{37}\text{H}_{67}\text{NO}_{13}$) of erythromycin mixed in the sample, a mass of 764.6370 was measured for the molecular ion of DGTS. This value is in agreement with the formula $\text{C}_{46}\text{H}_{86}\text{NO}_7$ (4.5 ppm error from the calculated mass of 764.6404 for the $[\text{M}+\text{H}]^+$ ion of a *N*-trimethyl homoserine betaine lipid containing two acyl functions corresponding to a total fatty acid composition of 36:2, *e.g.* two vaccenoyl residues. Vaccenic acid was the predominant fatty acid in DGTS as determined by GC-analysis. The mass spectrum revealed a fragment at m/z 500 resulting from the loss of water and one 18:1 acyl group. An abundant fragment at m/z 236 of the MS/MS spectrum was interpreted as the result of a loss of two water molecules and 25 two 18:1 acyl chains. A third fragment at m/z 162 representing the betaine head group, most likely resulted from elimination of glycerol minus water (propenediol) from the fragment m/z 30

236. Negative ion mode FAB-MS of DGTS was unsuccessful, as would be expected from a molecule carrying a net positive charge.

H-NMR analyses were performed in a Varian VXR500 spectrometer (500 MHz for protons) at 25°C, in CD₃OD for the glycolipid and in CD₃OD/CDCl₃ (1:1 vol/vol) for the
5 betaine lipid. The concentrations for the lipids were approximately 1 mg/ml. One-dimensional
¹H-spectra were measured using a 30-90° tipping angle for the pulse and 0.2 seconds as a
recycle delay between each of the 64 acquisitions. The chemical shifts are expressed in ppm
downfield from an external standard of Me₄Si and actually measured by reference to internal
CH₃OH (3.59 ppm) or CHCl₃ (7.24 ppm). Two-dimensional COSY- (¹H-¹H correlated
10 spectroscopy) and HMQC- (heteronuclear multiple quantum correlation spectroscopy) spectra
were recorded using standard procedures.

Briefly, in order to confirm the structure of the betaine lipid accumulating in *R. sphaeroides*, a ¹H-NMR was recorded for the purified compound. This spectrum was found to be nearly identical to published ¹H-NMR spectra for *N*- trimethylhomoserine betaine lipid
15 purified from the fern *Adiantum capillus-veneris* L. or the unicellular algae *Dunaliella parva* with the exception of the complexity for the fatty acid specific resonances due to different acyl groups in the different samples. The ¹H-NMR spectra for DGTS showed resonance values for the fatty acyl chains (0.6-2.5 ppm) and the glycerol protons (H-2 5.12 ppm, H-1_a 4.35 ppm, H-1_b 4.13 ppm, H-3_a 3.59 ppm, and H-3_b 3.55 ppm), thereby suggesting a diacylglycerol structure for
20 the lipid. The protons of the *N,N,N*-trimethyl group gave rise to a strong resonance at 3.18 ppm typical for all betaine lipids (See Sato, N., and Furuya, M., *Plant Cell Physiol.*, 21: 1113-1120 (1983); Evans *et al.*, *Chem. Phys. Lipids*, 31: 331-338 (1982); and Vogel *et al.*, *Chem. Phys. Lipids*, 52: 99-109 (1990)).

25

II. Expression of the *R. sphaeroides btaA* and *btaB* genes in Plants

The present invention also contemplates the expression of the *R. sphaeroides btaA* and *btaB* genes in plants. Although the present invention is not limited to the expression of said genes in any specific plant, in one embodiment, the expression of the *R. sphaeroides btaA* and
30 *btaB* genes in *Arabidopsis* is provided as follows.

A. Cloning and Expression of the *R. sphaeroides* *btaA* and *btaB* genes in Transgenic Plants

Transfer and expression of transgenes in plant cells is now routine practice to those skilled in the art. It has become a major tool to carry out gene expression studies and to attempt 5 to obtain improved plant varieties of agricultural or commercial interest. The present invention is not limited to the expression of the recombinant *R. sphaeroides* peptides encoded by SEQ ID NO: 1 and SEQ ID NO: 2 in bacteria and yeast. The invention also contemplates the expression of recombinant *R. sphaeroides* *btaA* (SEQ ID NO: 1) and *btaB* (SEQ ID NO: 2) genes in transgenic plants through agrobacterial transformation as described by S. Clough and A. Bent, 10 "Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*," *Plant J.*, 16: 735-43 (1998).

In one embodiment, the general process for manipulating genes to be transferred into the genome of plant cells to result in the expression of a recombinant peptide is carried out in two phases. First, all the cloning and DNA modification steps are done in *E. coli*, and the plasmid 15 containing the gene construct of interest is transferred by conjugation into Agrobacterium. Second, the resulting Agrobacterium strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in Agrobacterium and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to 20 Agrobacterium for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria (e.g., streptomycin), and the other for selection in plants (e.g., a gene encoding for kanamycin resistance or a gene encoding for resistance to an herbicide such as hygromycin). Also present are restriction endonuclease sites for the addition of one or more transgenes operably linked to appropriate regulatory sequences and directional T-DNA 25 border sequences which, when recognized by the transfer functions of Agrobacterium, delimit the region that will be transferred to the plant.

In another embodiment, plant cells may be transformed by shooting into the cell, tungsten microprojectiles on which cloned DNA is precipitated. (See, e.g., Gordon-Kamm *et al.*, *Plant Cell*, 2: 603 (1990)). In one embodiment, the Biolistic Apparatus (Bio-Rad, Hercules, 30 Calif.) is used for the shooting with a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast driving a plastic macroprojectile through a gun barrel. An aliquot of a

suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too small for the macroprojectile to go through. As a result, the plastic macroprojectile smashes against the stopping plate and the tungsten microprojectiles continue toward their target

5 through the hole in the plate. For the present invention the target can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

It is not intended that the present invention be limited to the particular manner by which the expression of any specific recombinant *R. sphaeroides* peptide in plants is achieved. In one embodiment, a peptide encoded by the nucleic acid sequences as set forth in SEQ ID NO: 1 is expressed in plants. In another embodiment, a peptide encoded by the nucleic acid sequence as set forth in SEQ ID NO: 2 is expressed in plants. In a further embodiment, two recombinant *R. sphaeroides* peptides encoded by the group of nucleic acid sequences comprising SEQ ID NO: 1 and SEQ ID NO: 2 are co-expressed in plants.

15 It is not intended that the present invention be limited by any particular plant cell type in which to generate the expression of recombinant *R. sphaeroides* gene products. In one embodiment, the plant cell is derived from a monocotyledonous plant. In an alternative embodiment, the plant cell is derived from a dicotyledonous plant. In another embodiment, the plant cell is derived from a group comprising the genera *Anacardium*, *Arachis*, *Asparagus*,

20 *Atropa*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Carthamus*, *Cocos*, *Coffea*, *Cucumis*, *Cucurbita*, *Daucus*, *Elaeis*, *Fragaria*, *Glycine*, *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*, *Hyoscyamus*, *Lactuca*, *Linum*, *Lolium*, *Lupinus*, *Lycopersicon*, *Malus*, *Manihot*, *Majorana*, *Medicago*, *Nicotiana*, *Olea*, *Oryza*, *Panicum*, *Pannisetum*, *Persea*, *Phaseolus*, *Pistacia*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Ricinus*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Sorghum*, *Theobromus*,

25 *Trigonella*, *Triticum*, *Vicia*, *Vitis*, *Vigna*, and *Zea*. In a preferred embodiment, the plant cell is derived from *Arabidopsis thaliana*.

B. Detection of Betaine Lipid Production in Plants

It is not intended that the present invention be limited to any specific method of detecting

30 the production of betaine lipids (including but not limited to DGTS) in plants. In one embodiment, the production of betaine lipids in plants is monitored by TLC as described above.

In another embodiment, said detection comprises the isolation of plant nucleic acids and Northern Blot Hybridization Analysis as described below.

1. Isolation of Total RNA from *Arabidopsis thaliana* Tissues

5 It is not intended that the invention be limited by any specific method to isolate total RNA from *A. thaliana* tissues. In one embodiment, total RNA is isolated from said tissues by guanidine hydrochloride extraction as follows. Said tissues are frozen in liquid nitrogen and homogenized to a fine powder using a Waring blender. For small amounts of tissue (less than 0.5 g), a rotating pin in a 1.5-ml Eppendorf tube is used to homogenize the tissue. The extract is
10 homogenized further at room temperature by the addition of 2 volumes of a guanidine buffer comprising 8 M guanidine hydrochloride, 20 mM MES (4-morpholineethansulfonic acid), 20 mM EDTA, and 50 mM 2-mercaptoethanol at pH 7.0.

The guanidine hydrochloride extract is centrifuged in a precooled (4°C) centrifuge for 10 minutes at 10,000 rpm. Subsequently the RNA-containing supernatant is filtered through one
15 layer of cheesecloth to get rid of floating particles. At least 0.2-1.0 vol of phenol/chloroform/IAA is added to extract proteins. After extraction the mixture is centrifuged for 45 minutes at 10,000 rpm at room temperature to separate the phases. The RNA-containing aqueous phase is collected and mixed with pre-cooled 0.7 volumes of ethanol and 0.2 volumes of 1 M acetic acid for precipitating the RNA and leaving DNA and residual proteins in the
20 supernatant. An overnight incubation at -20°C, or a 1 hour incubation at -70°C, is recommended.

The precipitated RNA is pelleted at 10,000 rpm for 10 min and washed twice with sterile 3 M sodium acetate at pH 5.2 at room temperature. Low-molecular-weight RNAs and contaminating polysaccharides dissolve, whereas intact RNA stays as a pellet after
25 centrifugation for 5 minutes at 10,000 rpm. The salt is removed by a final wash with 70% ethanol and the RNA pellet is subsequently dissolved in sterile water and stored at 20°C until needed. In the event that the total RNA isolated as described above requires further enrichment and purification prior to Northern Blot Hybridization Analysis, said RNA may be subjected to Poly A+ mRNA isolation as described below.

2. Poly A+ mRNA Isolation from *Arabidopsis thaliana* Total RNA

The present invention is not limited to any specific means of isolating Poly A+ mRNA from the total RNA of *Arabidopsis thaliana* leaves. In one embodiment, Poly A+ mRNA was isolated from *A. thaliana* leaf total RNA with the Oligotex mRNA Mini Kit (QIAGEN Cat. No. 5 70022) following the manufacturer's instructions as follows.

The Oligotex Suspension is heated to 37°C in a heating block, mixed by vortexing, and placed at room temperature. A sample containing 0.25 mg of *A. thaliana* leaf total RNA is pipetted into an RNase-free 1.5-ml microcentrifuge tube, and the volume of the reaction is adjusted to 0.25 ml with RNase-free water. A volume of 0.25 ml of Buffer OBB and 0.015 ml 10 of Oligotex Suspension are added to the reaction. The contents are mixed thoroughly by pipetting. The sample incubated for 3 minutes at 70°C in a water bath or heating block in order to disrupt secondary structure of the RNA. The sample is removed from the heating block, and placed at room temperature (20 to 30°C) for 10 minutes to allow hybridization between the oligo dT30 of the Oligotex particle and the poly-A tail of the mRNA. The Oligotex:mRNA complex 15 is pelleted by centrifugation for 2 minutes at maximum speed (14,000–18,000 x g), and the supernatant is removed by pipetting.

The Oligotex:mRNA pellet is resuspended in 400 µl Buffer OW2 by vortexing, and pipetted onto a small spin column supplied with the kit. The spin column is centrifuged for 1 minute at maximum speed (14,000–18,000 x g). The spin column is transferred to a new 20 RNase-free 1.5-ml microcentrifuge tube, and 400 µl of Buffer OW2 is applied to the column. The spin column is centrifuged for 1 minute at maximum speed and the flow-through fraction is discarded.

The spin column is transferred to another 1.5-ml microcentrifuge tube. A volume of 20–100 µl hot (70°C) Buffer OEB is pipetted onto the column. The resin is resuspended by 25 pipetting up and down three or four times to allow elution of the mRNA, and centrifuged for 1 minute at maximum speed to pellet the suspension. The flow-through fraction, which contains the Poly A+ mRNA is isolated and stored at -20°C until it is used.

3. Northern Blot Hybridization Analysis

Although the present invention is not limited to any specific method of performing Northern Blot analysis for detecting the production of betaine lipids (including but not limited to DGTS) in plants, in one embodiment, said analysis is performed as follows.

5 Prior to preparation of an agarose gel for Northern Blot Analysis, the electrophoresis chamber, gel tray, and gel comb are soaked in 1:10 diluted bleach for 30 min to 1 h. An agarose gel comprising 2.25 g of Agarose, 110 ml of H₂O, 15 ml of 10X MEN buffer (10X MEN: 41.9 g MOPS-NaOH, 4.1 g NaOAc, 3.72 g EDTA, pH 7.0, H₂O to 1000 ml, DEPC, autoclaved), 25 ml of 37% formaldehyde (Merck Cat.No. 3999) is prepared. The gel is poured into a 14 cm x 14 cm gel tray, and a 10-14 sample comb is inserted into the agarose gel.

10 Total RNA samples from plant tissue, isolated as described above, is prepared for electrophoresis as follows. For each sample, approximately 10 to 20 mg of RNA (in a volume of 20 µl) is mixed with 4 µl 10x MEN, 6 µl 37% formaldehyde, 20 µl fresh formamide, 0.5 µl 10 mg/ml ethidium bromide, 0.5 µl 1 mg/ml bromophenol blue (in DEPC-treated water) to a 15 total sample volume of 51.1 µl. The samples are incubated for 10 min at 56°C, and then placed on ice until loaded into the agarose gel. The samples are loaded into the gel sample wells prior to adding 1000 ml of electrophoresis buffer (1X MEN). The gel is electrophoresed at 100 Volts (constant voltage). After 15 min, when the samples have entered the gel (ca. 1 cm), the gel is submerged in the 1X MEN buffer. The gel is run at 100 Volt, for 3 to 5 h, until the blue dye has 20 migrated up to 2/3 of the gel. The gel is removed from the electrophoresis chamber and photographed.

25 The Northern Blot of the gel assembly is prepared by placing one 14 cm x 14 cm sheet of hybridization membrane (Hybond N+ from Amersham), two 14 cm x 14 cm sheets of filter paper (Whatman 3MM), and two 15 cm x 25 cm sheets of filter paper, on top of the gel. The sequence of placement of the filter papers and membrane are as follows. Prior to placement on the gel, the filter papers are moistened with 10X SSC, and the hybridization membrane is soaked in distilled water. The gel chamber is filled with 500 ml 10X SSC. The two 15 cm x 25 cm sheets of filter paper are placed in the gel chamber, and the agarose gel is placed upside-down on top of the sheets. The hybridization membrane is placed on top of the gel. The two 14 cm x 30 14 cm sheets of filter paper are placed on top of the membrane. Finally, paper towels are placed on top of the 14 cm x 14 cm sheets of filter paper, and a piece of plastic (e.g. gel tray) is placed

on top of the assembly with a glass bottle (100 to 500 g) to act as a weight. The assembly is left to blot the RNA from the gel overnight. Note that 10X SSC buffer may be prepared by making a 1:2 dilution of 20X SSC (175.3 g NaCl, 88.2 g Na citrate, pH 7.0, distilled water to 1000 ml, DEPC-treated and autoclaved) in DEPC-treated water.

5 The next day, the membrane is removed from the gel and marked in the upper right corner with the date. The membrane is air-dried for 15 min. The membrane is fixed incubating in 50 mM NaOH for 5 min. Alternatively, the membrane may be baked for 2 h at 80°C in a vacuum oven. Prior to pre-hybridization, the membrane is washed in 2X SSC for 2 min.

10 The membrane is placed in a 30 cm hybridization tube (Biometra) with pre-hybridization buffer comprising 250 mM Na_xPO₄, pH 7.4, 7% Sodium dodecyl sulfate (SDS), 1 mM EDTA, 1% Bovine Serum Albumin (BSA), 150 µl of a 10 mg/ml herring sperm DNA solution (denatured at 95°C for 3 min), and distilled water. The membrane is allowed to pre-hybridize for at least 4 hours prior to hybridization at 68°C.

15 Prior to hybridization, a radio-labeled hybridization probe is prepared as follows. A DNA fragment comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 1 (*btaA*) and SEQ ID NO: 2 (*btaB*), and portions thereof, is labeled by random-priming with fresh ³²P-dCTP (58 µCi, 3000 Ci/mol) using the Megaprime DNA labeling kit (Amersham Cat. No. RPN 1606-5-6-7) as per the manufacturer's instructions. The radio-labeled probe is added directly to the hybridization tube containing the pre-hybridization solution and membrane.

20 The membrane is allowed to hybridize overnight at 68°C.

25 Upon completion of hybridization, the membrane is washed 2X SSC, 0.1% SDS at 68°C in the hybridization tube for 5 min. The membrane is then removed from the tube and washed, in a glass or plastic container, 2-3 additional times for approximately 15 min each until the wash buffer is no longer radioactive. Once washing is completed, the membrane is placed in a plastic bag and exposed to X-ray film in a film cassette with an intensifying screen for 12-72 hours at -70°C. RNA samples which contained sequences homologous to the radio-labeled probe are visualized upon development of the X-ray film. A positive signal indicates that the plant from which the RNA sample was isolated produces RNA transcripts homologous to a nucleotide sequence selected from the group consisting of SEQ ID NO: 1 (*btaA*) and SEQ ID NO: 2 (*btaB*), and thus, indicates that the plant produces betaine lipids including, but not limited to, DGTS.

III. Method for the Production of DGTS *in vitro*

The methods of the present invention comprise the utilization of compositions comprising isolated and purified DNA having an oligonucleotide sequence selected from the group consisting of the *R. sphaeroides btaA* (SEQ ID NO: 1) and *btaB* (SEQ ID NO: 2) genes, 5 and portions thereof, such that DGTS is produced. In one embodiment, the production of the betaine lipid DGTS from a reaction mixture comprising isolated and purified protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and portions thereof, is contemplated.

In one embodiment, the *R. sphaeroides btaA* and *btaB* genes are cloned into pQE-31 and 10 pACYC-31, respectively, and expressed in *E. coli* as described above. Next, the *btaA* gene product (*i.e.* a first peptide), and the *btaB* gene product (*i.e.* a second peptide), are substantially purified using the QIAexpress Ni-NTA/6xHis-tag system as described above. Following purification of said peptides, DGHS (a betaine lipid precursor) is produced in a reaction containing means by reacting 50 mM Bicine (*N,N*-bis(2-hydroxyethyl) glycine), pH 8.1, 10 mM 15 MgCl₂, 1 mM cysteine, 100 μM *S*-adenosylmethionine (SAM), 100 μM diacylglycerol, and 10 μg of a substantially purified first peptide encoded by the amino acid sequence set forth in SEQ 20 ID NO: 3, in a 100 μl reaction volume at 37°C for 40 minutes. Next, 10 μg of a substantially purified second peptide encoded by the amino acid sequence set forth in SEQ ID NO: 4 and 100 μM SAM are added to the above reaction mixture such that DGTS is produced. (*See, e.g., Arondel et al., "Isolation and Functional Expression in Escherichia coli of a Gene Encoding Phosphatidylethanolamine Methlytransferase (EC 2.1.1.17) from Rhodobacter sphaeroides," J. Biol. Chem., 268(21): 16002-16008 (1993). In another embodiment, said first peptide is a gene product encoded by the nucleic acid sequence set forth in SEQ ID NO: 22 (M1-*btaA*). In another embodiment, said second peptide is a gene product encoded by the nucleic acid sequence set forth in SEQ ID NO: 23 (M1-*btaB*).*

The present invention is not limited by a specific means for verifying the production of DGTS by the method described above. The production of DGTS as a reflection of *S*-adenosylmethionine: diacylglycerol-3-amino-3-carboxyl transferase and *S*-adenosylmethionine:diacylglycerol homoserine-*N*-methyltransferase activity is detected by 30 Quantitative Lipid Analysis as described above. In another embodiment, the production of DGTS is verified by the following means. Aliquots of the above reaction are analyzed by thin

layer chromatography (TLC) on activated ammonium sulfate impregnated silica gel TLC plates with a solvent system containing acetone-toluene-water (91:30:8, vol/vol/vol). Products of the above reaction are then visualized with iodine vapor and identified by co-chromatography with an *R. sphaeroides* lipid extract known to contain DGTS.

5

IV. Variants of the Peptides Encoded by the *R. sphaeroides btaA* and *btaB* Genes

The present invention also contemplates variants of the peptides defined by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4. It is believed that the *R. sphaeroides btaA* (SEQ ID NO: 3) and *btaB* (SEQ ID NO: 4) genes can be "altered" at one or more selected codons to produce variants of the peptides encoded by said genes without significantly disrupting the wild-type functions of the peptides. An alteration is defined as a substitution, deletion, or insertion of one or more codons in the gene encoding the peptide of interest that results in a change in the amino acid sequence of the peptide as compared with the unaltered or wild-type sequence of the peptide. Preferably, the alterations are by conservative substitution of at least one amino acid with another amino acid in one or more regions of the molecule.

For example, it is contemplated that an isolated replacement of a leucine with an isoleucine or valine, an alanine with a glycine, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*i.e.*, conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative substitutions are those that take place within a family of amino acids that are related in their side chains. Amino acids can be divided into four families: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine); (3) nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); and (4) uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In an alternative, yet similar fashion, the amino acid repertoire can be grouped as: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine histidine), (3) aliphatic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic (phenylalanine, tyrosine, tryptophan); (5) amide (asparagine, glutamine); and (6) sulfur

-containing (cysteine and methionine) (See e.g., Stryer ed., *Biochemistry*, 2nd ed., WH Freeman and Co.(1981)).

Thus, in certain embodiments, modifications of the peptides encoded by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4 are contemplated by the present invention. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTar software or GCG (Univ. of Wisconsin).

Whether a change in the amino acid sequence of a peptide defined by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4 results in a peptide useful for the production of betaine lipids can be readily determined by monitoring the level of production of said lipids by TLC as described above (*i.e.* if the function of either the *R. sphaeroides* btaA or btaB peptide is significantly disrupted by the amino acid substitution, then the production of betaine lipids (*e.g.* DGTS) is reduced or completely abolished, and the TLC assay should reflect the difference in betaine lipid production when compared to such lipids produced by the wild-type *R. sphaeroides* peptides).

Oligonucleotide-mediated, or site-directed, mutagenesis is the preferred method for preparing substitution, deletion, or insertion variants of the peptides defined by the amino acid sequence of SEQ ID NO: 3 and SEQ ID NO: 4. The technique is well known in the art as described by Zoller *et al.*, *Nucl. Acids Res.*, 10: 6487 (1987). (See also Carter *et al.*, *Nucl. Acids Res.*, 13: 4331 (1986)).

Generally, oligonucleotides of at least 25 nucleotides in length are used. Although smaller oligonucleotides can be employed, an optimal oligonucleotide has 12 to 15 nucleotides that are complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide hybridizes properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea *et al.*, *Proc. Natl. Acad. Sci. USA*, 75: 5765 (1978).

The DNA template can only be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Vieira and Messing, *Meth. Enzymol.*, 153: 3-11 (1987). A preferred vector is pBlueScript II

SK+ (Stratagene) which contains the filamentous phage f1 origin of replication, thereby allowing the rescue of single-stranded DNA upon co-infection with a helper phage. Thus, the DNA that is to be mutated must be inserted into one of these vectors in order to generate single-stranded template. Production of the single-stranded template is described in sections 4.21-4.41 of Sambrook *et al.*, "Molecular Biology: A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989).

Briefly, in one embodiment, the *R. sphaeroides* wild-type *btaA* and *btaB* genes are altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template under suitable hybridization conditions, wherein the template is the single-stranded form of the plasmid containing the unaltered or wild-type DNA sequence for *btaA* (SEQ ID NO: 1) or *btaB* (SEQ ID NO: 2). After hybridization, a DNA polymerizing enzyme (*e.g.* the Klenow fragment of DNA polymerase I) is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis, and thus incorporates the oligonucleotide primer and codes for the selected alteration in the *btaA* and *btaB* genes. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the *btaA* or *btaB* gene, and the other strand (the original template) encodes the wild-type, unaltered sequence of the *btaA* or *btaB* gene. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened by colony hybridization using the oligonucleotide primer radio-labeled with 32 -Phosphate to identify the bacterial colonies that contain the mutated DNA. (See Short *et al.*, *Nucleic Acids Res.*, 16: 7583-7599 (1988)).

The method described immediately above can be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(AS) (which can be obtained from Amersham). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA contains dCTP-(AS) instead of dCTP, which serves to protect it from restriction endonuclease digestion. After the

template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA

5 homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

It is not intended that the present invention be limited to variants of the peptides defined by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID
10 NO: 4 wherein only a single amino acid substitution has been made. The present invention also contemplates variants that comprise greater than one amino acid substitution. Variants with more than one amino acid to be substituted can be generated in one of several ways. In one embodiment, if the amino acids are located close together in the polypeptide chain, they can be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid
15 substitutions. However, if the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods can be employed.

In another embodiment, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA
20 simultaneously, and the second strand of DNA that is synthesized form the template encodes all of the desired amino acid substitutions. An alternative embodiment involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA
25 molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be
30 used as a template in a third round of mutagenesis, and so on.

Transformation of prokaryotic cells is readily accomplished using the calcium chloride method as described in section 1.82 of Sambrook *et al.*, *supra*. Alternatively, electroporation (Neumann *et al.*, *EMBO J.*, 1: 841 (1982)) can be used to transform these cells. The transformed cells are selected by growth on an antibiotic, commonly tetracycline, kanamycin, or 5 ampicillin, to which they are rendered resistant due to the presence of *tet*, *kan*, and/or *amp* resistance genes on the vector.

Suitable prokaryotic host cells include *E. coli* strain JM101, *E. coli* K12 strain 294 (ATCC number 31,446), *E. coli* strain W3110 (ATCC number 27,325), *E. coli* X1776 (ATCC number 31,537), *E. coli* XL-1Blue MRF' (Stratagene), and *E. coli* B; however, many other 10 strains of *E. coli*, such as HB101, NM522, NM538, and NM539, and many other species and genera of prokaryotes can be used as well. In addition to the *E. coli* strains listed above, bacilli such as *Bacillus subtilis*, other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species can all be used as hosts.

After selection of the transformed cells, these cells are grown in culture and the plasmid 15 DNA (or other vector with the foreign gene inserted) is then isolated. Plasmid DNA can be isolated using methods known in the art. Two suitable methods are the small-scale preparation of DNA and the large-scale preparation of DNA as described in sections 1.25-1.33 of Sambrook *et al.*, *supra*. The isolated DNA can be purified by methods known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*. This purified plasmid DNA is then 20 analyzed by restriction mapping and/or DNA sequencing to confirm the presence of the desired *btaA* or *btaB* mutation in the selected transformant. DNA sequencing is generally performed by either the method of Messing *et al.*, "A system for shotgun DNA sequencing," *Nucleic Acids Res.*, 9: 309 (1981), the method of Maxam A.M. & Gilbert W., "Sequencing end-labelled DNA with base-specific chemical cleavages," *Meth. Enzymol.*, 65: 499-560 (1980), or the method of 25 Sanger *et al.*, "DNA sequencing with chain-terminating inhibitors," *Proc. Natl. Acad. Sci. USA*, 74: 5463-5467 (1977).

V. *R. sphaeroides btaA* and *btaB* Gene Homologs

It is not intended that the present invention be limited to the utilization of peptides 30 encoded by the oligonucleotide sequence of the *btaA* (SEQ ID NO: 1) and *btaB* (SEQ ID NO: 2) genes from *R. sphaeroides*. The present invention also relates to methods for discovering

homologs of the *R. sphaeroides* *btaA* and *btaB* genes in other organisms, and compositions comprised thereof. In one embodiment, the present invention contemplates utilizing compositions comprising peptides encoded by an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 23. In one embodiment, a method for the isolation and purification of the *R. sphaeroides* *btaA* and *btaB* gene homologs from *Mesorhizobium loti*, Ml-*btaA* (SEQ ID NO: 22)(Figure 22) and Ml-*btaB* (SEQ ID NO: 23)(Figure 23), is conducted as follows.

In order to identify *R. sphaeroides* *btaA* and *btaB* gene homologs in *M. loti*, the GenBank database of nucleic and amino acid sequences for *M. loti* (GenBank Accession Nos. AP002997, BA000012) is searched with the oligonucleotide and amino acid sequences of the *R. sphaeroides* *btaA* and *btaB* genes using TBLASTN. Two such homologs were identified in the organism *Mesorhizobium loti* (*i.e.* Ml-*btaA* and Ml-*btaB*), and an amino acid consensus alignment was performed. (*See Figures 20 and 21*). The identified homologs are then cloned into pPCR-Script Amp for expression in *E. coli* using a PCR-based strategy.

Briefly, genomic DNA from *M. loti* is isolated as described above for the isolation of *R. sphaeroides* genomic DNA. *M. loti* genomic DNA is then subjected to PCR in order to clone the Ml-*btaA* and Ml-*btaB* genes into *E. coli*. For example, for the Ml-*btaA* gene, a forward primer having the oligonucleotide sequence 5'- ACA TGC ATG CAA TGA CGG ACG TCT CCT CGG A-3' (SEQ ID NO: 24), and a reverse primer having the oligonucleotide sequence 5'- CGG GGT ACC TCA TGC CGT GCG CTT CAC AT-3' (SEQ ID NO: 25), are used such that *Sph* I and *Kpn* I sites, respectively, were generated. For the Ml-*btaB* gene, a forward primer having the oligonucleotide sequence 5'-GCG GAT CCG ATG ACC GAG CTG CCG G-3' (SEQ ID NO: 26), and a reverse primer having the oligonucleotide sequence 5'-GCA AGC TTT TAG CTG GCG ATC TTG ATC A-3' (SEQ ID NO: 27), are used such that *Bam* HI and *HinD* III sites, respectively, were generated.

Specifically, the Ml-*btaA* and Ml-*btaB* genes are generated by PCR in reaction mixtures (50 µl) comprising: 1X PCR buffer (Gibco-BRL); 2.5 mM MgCl₂ (Gibco-BRL); 350 nM forward primer; 350 nM reverse primer; 10% (v/v) DMSO; 200 µM dATP, dGTP, dCTP, dTTP; 1 ng *M. loti* genomic DNA; and 2.5 U Taq DNA polymerase (Roche Molecular). PCR reaction mixtures are subjected to thermal cycling in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, CA: Cat. No. N801-0001) under the following conditions: 1

denaturation cycle at 95°C for 3 minutes; 30 cycles comprised of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds; and 1 extension cycle at 72°C for 5 minutes. PCR products are run on 1% TAE agarose gel in the presence of ethidium bromide, and excised for purification by QIAEX II gel extraction kit (Qiagen Cat. No. 20021), followed by cloning into 5 the *SrfI* site of pPCR-Script Amp (as per manufacturer's instructions). The resulting plasmid constructs allow the independent expression of the recombinant *R. sphaeroides btaA* and *btaB* genes in *E. coli*.

Next, in order to isolate and purify the Ml-*btaA* and Ml-*btaB* gene products, the Ml-*btaA* and Ml-*btaB* genes are cloned into the protein expression vectors pQE-31 and pACYC-31. 10 Briefly, for the Ml-*btaA* gene, pQE-31 and pACYC-31 vectors are digested with *Sph I* and *Kpn I* and gel purified using the QIAEX II kit. The PCR insert is excised from pPCR-Script Amp by *Sph I/Kpn I* digest and gel purified, followed by ligation of the insert and vectors. Ligation reactions are then transformed into electrocompetent XL1-Blue *E. coli* and plated onto LB Ampicillin plates (pQE-31) or LB Chloramphenicol plates (pACYC-31). For the Ml-*btaB* 15 gene, pQE-31 and pACYC-31 are digested with *Bam HI* and *HinD III* and gel purified as described above. The insert is released from the vector by *Bam HI/HinD III* digest and gel purified, followed by ligation of insert and vectors. Ligation reactions are then transformed into electrocompetent XL1-Blue *E. coli* and plated onto LB Ampicillin plates (pQE-31) or LB Chloramphenicol plates (pACYC-31).

20 Each construct is analyzed individually for protein expression as detailed in the QIAexpress literature using M15[pREP4] as an expression host for the pQE-31 based plasmids and XL1-Blue as the host for pACYC-31 based constructs. Since the pACYC-31 and pQE-31 vectors carry compatible origins of replication, reconstitution of the DGTS biosynthetic pathway is achieved by the concurrent expression of pACYC-31:Ml-*btaA* and pQE-31:Ml-*btaB*, or 25 pACYC-31:Ml-*btaB* and pQE-31:Ml-*btaA*, in XL1-Blue cells. Cells expressing both of said genes are analysed by TLC for DGTS production after induction with IPTG as described above.

EXPERIMENTAL

EXAMPLE 1

In this example, a means for the amplification of the *R. sphaeroides* *btaA* and *btaB* genes, and their subsequent cloning into *E. coli*, is described. In one embodiment, the *btaA* gene was amplified from *R. sphaeroides* genomic DNA by PCR using a forward primer having the nucleotide sequence 5'-ACA TGC ATG CAG TGA CGC AGT TCG CCC TC-3' (SEQ ID NO: 5), and a reverse primer having the nucleotide sequence 5'-CGG GGT ACC AGG ACG ATC CGC TCG AAC CG-3' (SEQ ID NO: 6). The primers were used such that *BamH*I and *Hind*III sites were provided for cloning into pPCR-Script Amp (Stratagene Cat. No. 211188).

In another embodiment, the *btaB* gene was amplified using a forward primer having the nucleotide sequence 5'-ACA TGC ATG CAG TGA CGC AGT TCG CCC TC-3' (SEQ ID NO: 7), and a reverse primer having the nucleotide sequence 5'-CGG GGT ACC AGG ACG ATC CGC TCG AAC CG-3' (SEQ ID NO: 8). The primers were used such that *Sph* I and *Kpn* I sites were provided for cloning into pPCR-Script Amp.

All 50 µl PCR reaction mixtures contained the following: 1X PCR buffer (Gibco-BRL Cat. No. 18067-017), 2.5 mM MgCl₂, 350 nM forward primer, 350 nM reverse primer, 10% (v/v) dimethylsulfoxide (DMSO), 200 µM dATP, dGTP, dCTP, dTTP, 1 ng *R. sphaeroides* genomic DNA (isolated and purified as described above), and 2.5 U Taq DNA polymerase (Roche Molecular Cat. No. 1146173). PCR reaction mixtures were subjected to thermal cycling in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, CA: Cat. No. N801-0001) under the following conditions: 1 denaturation cycle at 95°C for 3 minutes; 30 cycles comprised of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds; and 1 extension cycle at 72°C for 5 minutes. PCR products were run on a 1% TAE agarose gel in the presence of ethidium bromide, and excised for purification by QIAEX II gel extraction kit (Qiagen Cat. No. 20021), followed by cloning into the *Srf*I site of pPCR-Script Amp (as per manufacturer's instructions). The resulting plasmid constructs allow the independent expression of the recombinant *R. sphaeroides* *btaA* and *btaB* genes in *E. coli*.

EXAMPLE 2

In this example, a method for the reconstitution of the betaine lipid biosynthetic pathway in plants is performed. The compositions and methods described herein provide for the

expression of the *R. sphaeroides* *btaA* and *btaB* genes targeted to the cytosol, or targeted to the plastid. Moreover, the compositions and methods described herein also provide for the selective expression of said genes only in seeds produced by the transformed plant, or alternatively, the constitutive expression of said genes in a transformed plant. All PCR reaction mixtures, thermal cycling program parameters, and component sources are as described above in Example 1.

a. Binary vectors for the constitutive expression of the *R. sphaeroides* *btaA* and *btaB* genes in the plant cytosol are prepared using a PCR-based strategy as follows. For this purpose, the *btaA* gene sequence was amplified by PCR using a forward primer having the nucleotide sequence 5'-GCT CTA GAA TGG CGC AGT TCG CCC TC-3' (SEQ ID NO: 11), and a reverse primer having the nucleotide sequence 5'-ACA TGC ATG CAG GAC GAT CCG CTC GAA CCG-3' (SEQ ID NO: 12). The *btaB* gene sequence was amplified by PCR using a forward primer having the nucleotide sequence 5'-GCT CTA GAA TGG CCG ACG CCA CCC AT-3' (SEQ ID NO: 13), and a reverse primer having the nucleotide sequence 5'-ACA TGC ATG CAG GAC GAT CCG CTC GAA CCG-3' (SEQ ID NO: 14). The primers were constructed such that *Sph* I and *Xba* I sites are provided for subsequent cloning of the *btaA* and *btaB* gene PCR products into the corresponding restriction sites on the binary vector, pBinAR-Hyg. This vector is derived from pBIB-Hyg (Becker, D., *Nucleic Acids Res.* 18: 203 (1990)) by insertion of the *Hind* III-*Eco* RI fragment from the central portion of pA7 (von Schaeven, A., Ph.D. thesis, Freie Universität Berlin (1989)). This construct is introduced into *Agrobacterium tumefaciens* strain C58C1 and used to transform *Arabidopsis thaliana* Col-2 plants as described below.

b. Binary vectors for the constitutive expression of the *R. sphaeroides* *btaA* and *btaB* genes targeted to the plastid are prepared using a two-stage Splicing by Overlap (SOE)-PCR-based strategy as follows. In the first stage, the *btaA* gene sequence was amplified by SOE-PCR using a forward primer having the nucleotide sequence 5'-ATG CAG GTG TGG CCT CCA GTG ACG CAG TTC GCC CTC-3' (SEQ ID NO: 15), and a *rbcS*-specific reverse primer having the nucleotide sequence 5'-GAG GGC GAA CTG CGT CAC TGG AGG CCA CAC CTG CAT-3' (SEQ ID NO: 16). The *btaB* gene sequence was amplified by SOE-PCR using a forward primer having the nucleotide sequence 5'-ATG CAG GTG TGG CCT CCA

ATG ACC GAC GCC ACC CAT-3' (SEQ ID NO: 17), and *rbcS*-specific reverse primer having the nucleotide sequence 5'-ATG GGT GGC GTC GGT CAT TGG AGG CCA CAC CTG CAT-3' (SEQ ID NO: 18). The *rbcS*-specific primers are used to fuse the *rbcS* transit peptide from the Pea ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) (GenBank Accession No. 5 X04333), as described by Fluhr *et al.*, "Expression and dynamics of the pea *rbcS* multigene family and organ distribution of the transcripts," *EMBO J.*, 5: 2063-2071 (1986), to the *btaA* and *btaB* gene sequences individually. After amplification, the *rbcS*, *btaA*, and *btaB* PCR products are gel purified using QIAEX II (QIAGEN).

In the second stage, the purified *rbcS*, *btaA*, and *btaB* PCR products are then subjected to 10 a second round of PCR. For the *btaA* gene, a *rbcS*-specific forward primer having the nucleotide sequence 5'-GCT CTA GAA ACC ACA AGA ACT AAG AA-3' (SEQ ID NO: 19), and a reverse primer having the nucleotide sequence 5'-ACA TGC ATG CAG GAC GAT CCG CTC GAA CCG-3' (SEQ ID NO: 20), are used. For the *btaB* gene, a reverse primer having the nucleotide sequence 5'-ACA TGC ATG CCT CTC ACC GCG TGA GCG TG-3' (SEQ ID NO: 15 21), and the same *rbcS*-specific forward primer (SEQ ID NO: 19), are used.

The second-stage PCR primers were constructed such that *Sph* I and *Xba* I sites are provided for subsequent cloning of the *rbcS* transit peptide-fused *btaA* and *btaB* gene PCR products into the corresponding restriction sites on the binary vector, pBinAR-Hyg. However, prior to cloning into pBinAR-Hyg, said PCR products are cloned into pPCR-Script Amp and cut 20 with *Sph* I and *Xba* I. The plasmids containing the desired PCR products are transformed into *E. coli* and grown in LB medium with 50 mg/ml ampicillin. The plasmid DNA is isolated and digested with *Sph* I and *Xba* I, followed by gel purification of the desired plasmid inserts as described above. Finally, the inserts are sub-cloned into the corresponding sites of pBinAR-Hyg. The resulting plasmid constructs are introduced into *Agrobacterium tumefaciens* strain C58C1 25 and used to transform *Arabidopsis thaliana* Col-2 plants as described below.

c. Binary vectors for the seed-specific expression of the *R. sphaeroides* *btaA* and *btaB* genes in the plant cytosol and plastid are prepared using a PCR-based strategy as described above with the following substitutions. In order to obtain seed-specific expression of the *R. sphaeroides* *btaA* and *btaB* genes in plants, the binary vector pBinUSP-Hyg is used in place of 30 the pBinAR-Hyg vector described above. The pBinUSP-Hyg vector contains the USP promoter

derived from the broad bean plant as described by Fiedler *et al.*, "A complex ensemble of cis-regulatory elements controls the expression of a *Vicia faba* non-storage seed protein gene," *Plant Mol. Biol.*, 22: 669-679 (1993). The use of the USP promoter to obtain seed-specific expression of proteins has been demonstrated in *A. thaliana*. Bäumlein *et al.*, "A novel seed 5 protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants," *Mol. Gen. Genet.*, 225: 459-467 (1991). The resulting plasmid constructs are introduced into *Agrobacterium tumefaciens* strain C58C1 and used to transform *Arabidopsis thaliana* Col-2 plants as described below.

10 d. A means for the simplified transformation of *Arabidopsis* is described herein and follows the methods of S. Clough and A. Bent, "Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*," *Plant J.*, 16:735-43 (1998). Arabidopsis plants are grown under long days in pots in soil covered with bridal veil, window screen or cheesecloth, until they are flowering. First bolts are clipped to encourage proliferation 15 of many secondary bolts, causing the plants to be ready roughly 4-6 days after clipping. Optimal plants have many immature flower clusters and not many fertilized siliques, although a range of plant stages can be successfully transformed.

20 The *Agrobacterium tumefaciens* strain carrying the gene of interest on a binary vector is grown in a large liquid culture at 28°C in LB (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter of water) with 25 µg/ml hygromycin B (Calbiochem) to select for the binary plasmid. The Agrobacterium culture is pelleted by centrifugation at 5500 X g for 20 minutes, and resuspended to OD₆₀₀ = 0.8 in a sterile 5% Sucrose solution.

25 Before the above-ground parts of an Arabidopsis plant are dipped in the resuspended Agrobacterium/Sucrose solution, Silwet L-77 (OSi Specialties, Inc., Danbury, CT) is added to a concentration of 0.05% (500 µl/L) and mixed well. The above-ground parts of an Arabidopsis plant are dipped in the Agrobacterium solution for 2 to 3 seconds, with gentle agitation. The dipped plants are placed under a dome or cover for 16 to 24 hours to maintain high humidity. The dipped plants are not exposed to excessive sunlight as the air under the dome can get hot.

30 The plants are grown for a further 3-5 weeks and watered normally, tying up loose bolts with wax paper, tape, stakes, twist-ties, or other means. Watering is halted as the seeds of the plant become mature. Once mature, the dry seeds are harvested by the gentle pulling of grouped

inflorescences (*i.e.* flower clusters) through fingers over a clean piece of paper. The majority of the stem and pod material is removed from the paper and the seeds are stored under dessication at 4°C.

Successful transformants capable of expressing a recombinant *A. thaliana* peptide are selected by using an antibiotic or herbicide selectable marker. In this example, 2000 harvested seeds (resuspended in 4 ml 0.1% agarose) are vapor-phase sterilized and plated on selection plates with 50 µg/ml hygromycin B, cold treated for 2 days, and then grown under continuous light (50-100 µEinsteins) for 7-10 days. The selection plates of the example further comprise 0.5X Murashige-Skoog medium (Sigma Chem. Cat. No. M-5519) and 0.8% tissue culture Agar (Sigma Chem. Cat. No. A-1296). Successful transformants are identified as hygromycin-resistant seedlings that produce green leaves and well-established roots, within the selective medium.

A sample of successful transformants are grown to maturity by transplantation into heavily moistened potting soil. Leaves from the transformants are removed and subjected to DNA extraction to isolate the genomic DNA of the plant. The extracted genomic DNA is subsequently subjected to restriction endonuclease digestion and Southern Blotting to confirm the incorporation of the gene of interest into the plant's genome.

e. A method for the crossing of a transformed plant containing the *R. sphaeroides* *btaA* gene with a transformed plant containing the *R. sphaeroides* *btaB* gene, such that the betaine lipid biosynthetic pathway is reconstituted in a single plant, is provided as follows.

A transformed female parent plant (4-6 weeks-old) containing the *R. sphaeroides* *btaA* is used as a pistil donor. Several young flower buds that are located at the top of the inflorescence on the main flowering stalk are chosen. The newly emerging white petals should be barely visible in the most mature flower bud chosen. The use of any flower bud that has opened and potentially exposed its pistil to parental pollen or another pollen source is avoided. For example, a bud at the correct stage will contain short immature stamens with anthers that are greenish-yellow in color. All other flower buds and flowers from the inflorescence are removed.

Prior to dissection of the chosen flower buds, forceps are sterilized in 95% ethanol and air-dried to remove contaminating pollen. Next, the sepals, petals, and stamens are removed from the flower buds beginning with the tissue near the base of the flower bud. Great care

should be taken not to injure the pistil or flower stalk while dissecting the flower bud. When finished, the pistil is free of sepals, petals, and stamens.

A transformed male parent plant (4-6 weeks-old) containing *R. sphaeroides btaB* is used as a pollen donor. First, a suitable pollen-donor flower is selected. For example, for wild-type 5 Arabidopsis, a flower that has opened and has petals that are perpendicular to the main flower body is chosen. To confirm that the chosen flowers are in the process of releasing pollen, visual examination of the anthers from several flowers to identify the flowering stage associated with pollen release is performed. Next, the flower is removed from the flowering stalk, followed by removal of the petals and sepals from the flower. This process yields 6 stamens (2 short and 4 10 tall) for each flower. Several stamens are removed and their anthers checked for pollen. The pollen grains should be clearly visible when viewed under a dissecting microscope. When anthers brimming with pollen are identified, they are used to pollinate the stigmas of the previously prepared pistils. To maximize the probability of pollination, each pistil is pollinated with several anthers.

15 When pollination is complete, the pistil is covered with a small piece of plastic wrap (1cm x 1cm) to protect it from other pollen sources. The plastic wrap is folded in half around the pistil. Next, the pollinated pistil is marked by applying a small piece of tape describing the cross on the corresponding flowering stalk. The plastic wrap is removed in 1 to 2 days.

Following a successful pollination, the pistil elongates as the seeds develop. When the siliques 20 are fully elongated and have dried to a golden-brown color, they are removed from the plant, taking care not to shatter the siliques and lose the seeds. The seeds are allowed to dry for at least one week before planting. The seeds can also be chilled at 4°C for several days following imbibition to increase the frequency of germination. Germinated seeds are planted to produce plants which comprise both the *R. sphaeroides btaA* and *btaB* genes, thereby reconstituting the betaine lipid 25 biosynthetic pathway in a single plant. Lipid extracts may be made from transformed plant leaves and seeds, and subjected to quantitative lipid analysis by TLC (as described above) to confirm the production of betaine lipids including, but not limited to, DGTS.

EXAMPLE 3

30 In this example, one method of generating variants of the peptides defined by an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, by

conservative amino acid substitution is provided. Briefly, this method comprises the cloning of the *R. sphaeroides* *btaA* and *btaB* genes into the phagemid vector pBluescript II SK+, growth and recovery of single stranded DNA templates for each of said genes, oligonucleotide-directed mutagenesis, transformation of suitable host cells for production of double-stranded DNAs containing the directed mutation, and confirmation of the transformants as having incorporated the desired mutation. This method is performed as described in the manufacturer's instruction manual for the "pBlueScript II Exo/Mung DNA Sequencing System." (Stratagene Cat. No. 212301).

10 **a. Cloning of *btaA* and *btaB* into pBluescript II SK+**

The independent cloning of the *R. sphaeroides* *btaA* and *btaB* genes into the phagemid vector pBluescript II SK+ is accomplished as described above in Part I.A.

15 **b. Recovery of Single-Stranded DNA Template from Cells Containing
pBlueScript II SK+ Phagemids**

pBluescript II SK+ is a phagemid which can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. coli* with the F+ phenotype (containing an F' episome), pBluescript II SK+ phagemids will be secreted as single-stranded f1 "packaged" phage when the bacteria has been infected by a helper phage. Since these filamentous helper phages (VCSM13, f1) will not infect *E. coli* without an F' episome coding for pili, it is essential to use XL1-Blue MRF' (Stratagene Cat. No. 212301) or a similar strain containing the F' episome.

Typically, 30–50 pBluescript II SK+ molecules are packaged/helper phage DNA molecule. pBluescript II SK phagemids are offered with the IG region in either of two orientations: pBluescript II SK (+) is replicated so the coding strand of the β-galactosidase gene (the top strand in the enclosed map, the same strand as in the mp vectors) is secreted within the phage particles; pBluescript II SK (-) is replicated so the non-coding strand of the β-galactosidase gene is secreted in the phage particles.

Yields of single-stranded (ss)DNA depend on the specific insert sequence. For most inserts, over 1 µg of ssDNA can be obtained from a 1.5-ml miniprep if grown in XL1-Blue

MRF'. A faint single-strand helper phage band may appear on a gel at ~4 kb for R408 or 6 kb for VCSM13. This DNA mixture can be sequenced with primers that are specific for pBluescript II SK+ (e.g. the SK primer and M13(-20) primer (Stratagene Cat. Nos. 300305 & 300303, respectively)) and do not hybridize to the helper phage genome.

- 5 VCSM13 and R408 helper phage produce the largest amount of single-strand pBluescript II SK+. R408 (single-strand size ~4 kb) is more stable and can be grown more easily. VCSM13 (single-strand size ~6 kb), being more efficient, yields more single-stranded phagemid; however it is more unstable and reverts to wild-type more frequently. This difficulty can be addressed by periodically propagating VCSM13 in the presence of kanamycin. VCSM13 (a derivative of
10 M13KO7) has a kanamycin gene inserted into the intergenic region (IG), while R408 has a deletion in that region.

The advantages of using pBluescript II phagemids for site-specific mutagenesis using standard techniques are as follows: (1) pBluescript II SK phagemids do not replicate via the M13 cycle, lessening the tendency to delete DNA inserts, therefore it is unlikely that even 10-kb
15 inserts will be deleted; (2) "packaging" of pBluescript II SK phagemids containing inserts is efficient since the pBluescript II SK vector is 3.5 kb (smaller than wild-type M13); and (3) oligonucleotide mutagenesis in pBluescript II SK vectors is advantageous because the mutagenized insert is located between the T3 and T7 promoters. The resultant mutant transcripts can be synthesized *in vitro* without further subcloning.

20

c. Single-Stranded Template DNA Rescue Protocol

In one embodiment, single-stranded DNA template for oligonucleotide-mediated mutagenesis is prepared from pBlueScript II SK+ phagemids comprising an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, as follows.

- 25 A single colony containing pBlueScript II SK+ comprising an oligonucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, is inoculated into 5 ml of 2X YT containing 50 µg/ml ampicillin and VCM13 or R408 helper phage at 10^7 – 10^8 pfu/ml (multiplicity of infection ~10). The culture is grown at 37°C with vigorous aeration for 1–2 hours. If VCSM13 is used as the helper phage, kanamycin is added to the media at a
30 concentration of 70 µg/ml to select for infected cells. The cells are allowed to continue to grow at 37°C for 16–24 hours, or until growth has reached saturation. The cells are transferred to 1.5

ml microcentrifuge tubes and centrifuged for 5 minutes. Approximately 1 ml of supernatant is removed, 150 µl of a 20% PEG (polyethylene glycol)/2.5 M NaCl solution is added, and the phage particles are allowed to precipitate on ice for 15 minutes. The phage particles are centrifuged for 5 minutes in a microcentrifuge, followed by the removal of the supernatant. The
5 PEG/phage pellets are centrifuged for a few seconds more to collect residual liquid, which is subsequently removed. The pellets are resuspended in 400 µl of 0.3 M NaOAc (pH 6.0) and 1 mM EDTA by vortexing vigorously.

The resuspended pellets are extracted with 1 volume phenol:chloroform and centrifuged for 1–2 minutes to separate the aqueous and organic phases. The aqueous phase is transferred to
10 a fresh tube, 1 ml of 100% ethanol is added, and the tube is centrifuged for 5 minutes. The ethanol is removed , the DNA pellet is air-dried and dissolved in 25 µl of TE buffer. For analysis, 1–2 µl of the dissolved ssDNA template may be run on an agarose gel.

d. Oligonucleotide-Mediated/Site-Directed Mutagenesis

15 Single-stranded DNA templates from cells containing pBluescript II SK+ Phagemids are isolated (as described above) and used for oligonucleotide-mediated mutagenesis according to the following protocol as described in the instruction manual for the "pBluescript II Exo/Mung DNA Sequencing System" (Stratagene Cat. No. 212301). Briefly, oligonucleotides having a oligonucleotide sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6,
20 SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10 are hybridized to their corresponding ssDNA templates in order to induce mutagenesis as follows. Said oligonucleotides are designed to generate the following corresponding mutations in either the *R. sphaeroides btaA* or *btaB* gene sequence as indicated in the table below.

Table 3. Details of *R. sphaeroides* *btaA* and *btaB* Mutagenesis

Mutagenesis	Corresponding	<i>R. sphaeroides</i>	Amino Acid
Oligonucleotide	SEQ ID NO:	gene mutated	change generated
btaA-L9I	5	<i>btaA</i>	L9I
btaA-A201G	6	<i>btaA</i>	A201G
btaA-S399T	7	<i>btaA</i>	S399T
btaB-T13S	8	<i>btaB</i>	T13S
btaB-I115L	9	<i>btaB</i>	I115L
btaB-G206A	10	<i>btaB</i>	G206A

An oligonucleotide having a oligonucleotide sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10 is kinased in a reaction comprising: 100 ng of mutagenesis oligonucleotide; 4 μ l of 10X ligase buffer (500 mM Tris-HCl, pH 7.5; 70 mM MgCl₂; and 10 mM dithiothreitol (DTT)); 4 μ l of 10 mM rATP; 2 μ l of T4 polynucleotide kinase (10 U) (Promega Cat. No. M4101); and water to 40 μ l final volume. The reaction is incubated at 37°C for 30 minutes.

In order to synthesize a *btaA* or *btaB* variant DNA strand comprising the desired amino acid substitution (*i.e.* mutation), the kinased mutagenesis oligonucleotide is annealed to 1 μ g of ssDNA template in a reaction comprising 20 μ l of oligonucleotide from the kinase reaction above (50 ng) and 5 μ l of salmon sperm DNA. The reaction is incubated at 65°C for 10 minutes, then at room temperature for 5 minutes. Once the mutagenesis oligonucleotide has been annealed to the ssDNA template, the second strand of the DNA template (incorporating the amino acid substitution) is generated by primer extension as follows. To the annealing reaction, the following is added: 4.0 μ l of 10X ligase buffer (same as above); 2.0 μ l of 2.5 mM dNTPs (N = A, C, G and T in equal concentration); 4.0 μ l of 10 mM rATP; 1.0 μ g of single-stranded DNA binding protein (Promega Cat. No. M3011); 1.5 U of DNA Polymerase I, Klenow Fragment (Promega Cat. No. M2201); 0.5 μ l of T4 DNA ligase (2 U) (Promega Cat. No. M1801), and water to 40 μ l final volume. The reaction is incubated at room temperature for 3–4 hours.

After incubation is complete, *E. coli* XL1-Blue MRF' cells are transformed with 10 μ l of synthesis reaction and plated on LB medium (10 g NaCl, 10 g tryptone, 5 g yeast extract,

deionized water to 1000 ml, and adjusted to pH 7.0) plates containing 50 µg/ml ampicillin, 12.5 µg/ml tetracycline, 80 µg/ml freshly prepared X-Gal (Promega Cat. No. V3941), and 20 mM IPTG to allow antibiotic and blue-white color selection of transformed bacterial colonies containing phagemids comprising the desired amino acid change. Said colonies may be
5 screened for incorporation of the desired amino acid substitution by colony hybridization analysis as described below. If said colonies are to be screened by colony hybridization, then transformed XL1-Blue MRF' cells should be plated onto nitrocellulose filters placed on top of three LB plates lacking IPTG. After 8-10 hours of incubation, the nitrocellulose filters are transferred to LB plates containing 5 mM IPTG for several hours.

10

e. **Screening Transformant Colonies for Confirmation of Amino Acid Substitution**

Colonies containing pBluescript II SK+ phagemids may be screened for recombinants by many techniques widely known in the art such as double-stranded DNA, RNA, or
15 oligonucleotide hybridization (e.g. colony hybridization). (See Instruction Manual for the "pBluescript II Exo/Mung DNA Sequencing System" (Stratagene Cat. No. 212301)). Colonies may also be screened by restriction endonuclease mapping or by sequencing plasmid DNA (e.g. Sanger dideoxy chain terminator DNA sequencing, Maxam & Gilbert sequencing) to confirm the presence of an amino acid substitution at the desired amino acid residue.

20

EXAMPLE 4

In this example, one method of cloning and expressing *Bta1* from *Chlamydomonas reinhardtii* is provided.

Briefly, the protein sequence of BtaA from *Rhodobacter sphaeroides* (RsBtaA) was used
25 as a query in a TBLASTN search against a draft of the *C. reinhardtii* genome found at the Joint Genome Institute website of the Department of Energy. In this way, a protein with strong similarity to RsBtaA was identified. The predicted protein is encoded by a gene on scaffold 250 from about position 17000-23500. The protein predicted by the "green genie" prediction program is 648 amino acids long and contains an N-terminal region with significant identity to
30 the bacterial BtaB protein, and a C-terminal region similar to the bacterial BtaA protein. Thus, it is contemplated that a single *C. reinhardtii* protein is responsible for all reactions of DGTS

synthesis, and thus the newly-identified gene was given the name “*BtaI*” to reflect this. The genomic DNA sequence of *C. reinhardtii BtaI* is set forth herein as SEQ ID NO: 43, while the cDNA sequence is set forth as SEQ ID NO: 44, and the protein sequence (CrBta1) is set forth as SEQ ID NO: 45. Primers were designed to amplify the coding sequence by RT-PCR and to
5 facilitate expression of the protein as an N-terminal His-tag fusion in the pQE-31 expression vector (See, Figure 1). The forward primer contained a *Bam* HI site upstream of the start codon 5'-CAG GAT CCA ATG GGG TCG GGT CGT-3' (SEQ ID NO:46); while the reverse primer contained a *Kpn* I site upstream of the stop codon: 5'-CAG GTA CCG CCG CCA GCT GCT TA-3' (SEQ ID NO:47).

10 A *C. reinhardtii* cell-wall deficient mutant, CC-400, was grown to mid log phase in TAP medium, and harvested. A 50 ml culture aliquot was used for RNA isolation by TRIzol reagent (Invitrogen), and 2 µg total RNA was used in a reverse transcriptase reaction (SuperScript II RNase H⁻ Reverse Transcriptase) according to the manufacturer's instructions. Subsequently, a 2 µl aliquot of the RT reaction was used as a template in a 50 µl PCR reaction with the above
15 primers, using Pfu Turbo polymerase and Pfu Native Plus reaction buffer (Stratagene). Due to the high G+C content of *Chlamydomonas* DNA, DMSO was added to 10% (v/v). Primers were used at a concentration of 350 nm, and dNTPs were used at a concentration of 250 µM each. Cycling was as follows: 5 min at 95°C, then 35 cycles at 95°C for 30 sec; 54°C for 30 sec; and 72°C for 2 min; with a final extension step at 72°C for 10 min.

20 A product of approximately 1950 bp was produced and cloned into the *Eco* RV site of pBlueScript II KS+ to facilitate sequencing, then excised by *Bam* HI and *Kpn* I digestion in order to directionally clone the fragment into the corresponding sites of pQE-31, giving rise to plasmid pBta1. As shown in Figure 32, lipid extracts of cells harboring the pBta1 plasmid produced the lipid DGTS to a significant level, estimated at 10-15 mol % based on TLC iodine staining
25 intensity. DGTS was purified from *E. coli* expressing CrBta1 and analyzed by NMR. As shown in Figure 33, a strong resonance at ~3.2 ppm was detected, characteristic of the quaternary ammonium function of DGTS, thus confirming the identity of this product. In addition, the inventors contemplate subcloning CrBta1 into pBinAR-Hyg for expression in plants, as described above for *R. sphaeroides BtaA* and *BtaB* genes in Part II of the Description.

EXAMPLE 5

In this example, one method of cloning and expressing *Bta1* from *Neurospora crassa* is provided.

Briefly, a protein with domains sharing significant sequence similarity with the BtaA and
5 BtaB proteins of bacteria was identified in *Neurospora crassa* genome contig 3.153, by using three sequence analysis programs (combination of FGENESH, FGENESH+, and GENEWISE). The contig is part of the *N. crassa* 3 database found at the web site of the Whitehead Institute's Center for Genome Research. The protein encoded by the minus strand of the *N. crassa* contig (at about position 218593-221450 as set forth in SEQ ID NO: 48) has a N-terminal portion
10 similar to BtaB, and a C-terminal portion similar to BtaA. Thus, the inventors contemplate that the protein functions similarly to the Bta1 protein from *Chlamydomonas reinhardtii*, and hence the gene has been termed *N. crassa Bta1*. The coding region of the gene is provided herein as SEQ ID NO: 49, while the amino acid sequence of the predicted protein (NcBta1) is provided as SEQ ID NO: 50.
15 Cloning of the open reading frame by RT-PCR is accomplished using the following primer pair: forward, 5'-CAG GTA CCG GAT CCA ATA GCA ATG GGA GAC AAC-3' (SEQ ID NO: 51); and reverse, 5'-CAA AGC TTT CTA GAC TAC TTA AGC TGA GTC AAC C-3' (SEQ ID NO: 52). The *Kpn* I and *Bam* HI sites in the forward primer and the *HinD* III and *Xba* I sites in the reverse primer were introduced to facilitate cloning into pQE-31 for expression in *E. coli* and
20 pBinAR-Hyg for expression in plants, as described above for *R. sphaeroides BtaA* and *BtaB* genes in Parts I and II of the Description.

During development of the present invention, the DGTS lipid was found to be produced by *N. crassa* only during low phosphate conditions. Specifically, as shown in Figure 34, DGTS was produced by *N. crassa* when grown in a modified Vogel's medium containing MES and 0.01
25 mM Pi, but not when grown in Vogel's medium containing 20 mM Pi. A similar expression pattern was observed for NcBta1 RNA. A complete description of Vogel's medium can be found in Vogel, Microbiol. Genetics Bull., 13:42-43 (1956), herein incorporated by reference.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

5